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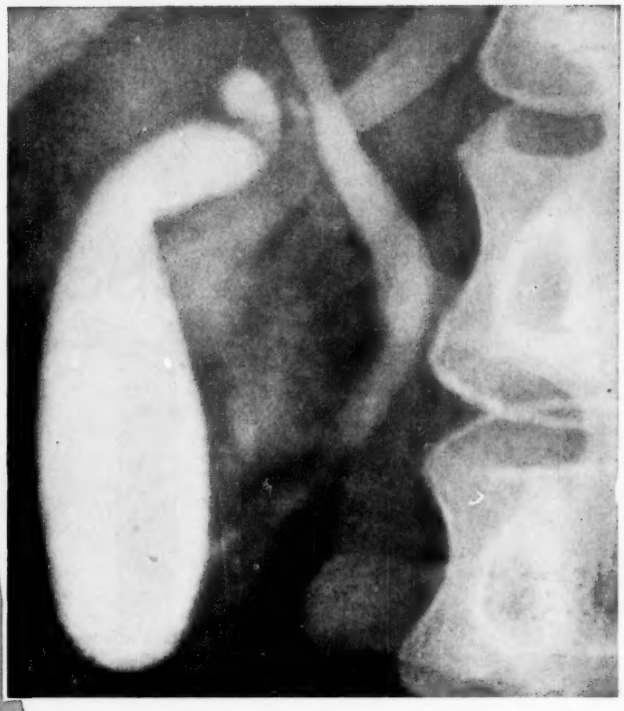

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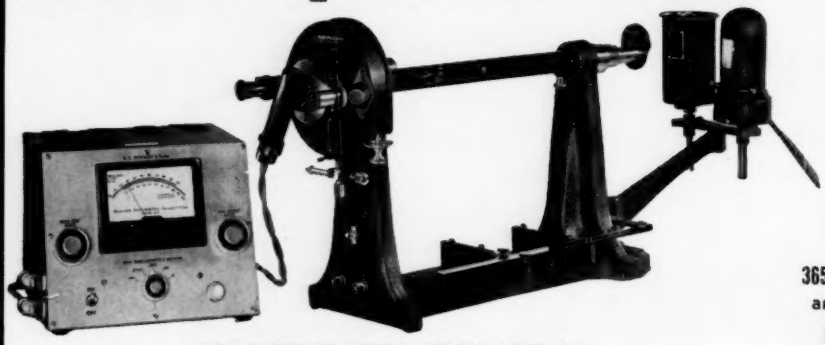

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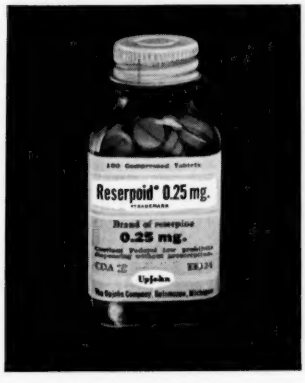

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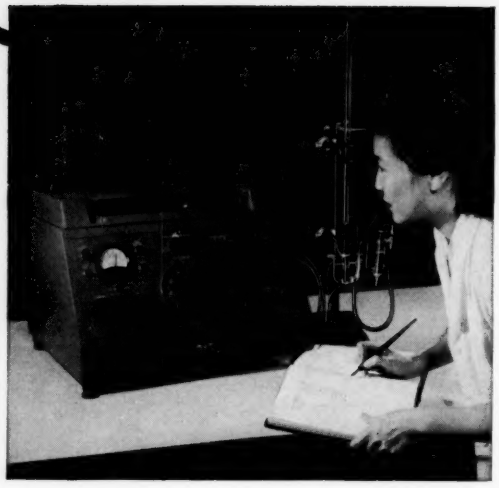

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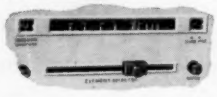

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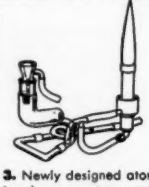

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ANALYSIS OF THE RESPIRATORY RESPONSE TO CARBON DIOXIDE INHALATION IN VARYING CLINICAL STATES OF HYPERCAPNIA, ANOXIA, AND ACID-BASE DERANGEMENT

By JAMES K. ALEXANDER,[1, 2, 3] JOHN R. WEST,† JOHN A. WOOD, AND DICKINSON W. RICHARDS

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Cardio-pulmonary Laboratory of the Presbyterian Hospital, New York City, N. Y.)

(Submitted for publication July 30, 1954; accepted December 1, 1954)

Individuals with cor pulmonale secondary to chronic pulmonary emphysema tend to have pulmonary ventilation which is less than normal both at rest and during exercise, despite the presence of factors ordinarily making for increased ventilation such as anoxemia and acidosis (1), and despite the fact that these ventilatory levels may be appreciably less than the observed maximum breathing capacity. Moreover, the ventilatory response to increased CO_2 in the inspired air may be less than normal in certain patients with pulmonary emphysema (2–5). These observations have been interpreted as indicating a disorder of the chemical regulation of respiration associated with emphysema.

The present study was undertaken to define more specifically the nature of this disorder in terms of the sensitivity of the neural regulatory mechanism to the carbon dioxide-hydrogen ion stimulus, and to characterize the conditions under which such a disorder might exist. Sensitivity of the neural regulatory mechanism to CO_2 and/or hydrogen ion has been evaluated quantitatively by correlation of arterial blood hydrogen ion concentration and CO_2 tension with effective alveolar ventilation when changes are brought about by varying amounts of carbon dioxide in the inspired air.

In the present investigation, the effects of CO_2 inhalation in normal subjects have been compared with those in emphysematous subjects having disease of varying severity.

In order to evaluate separately the possible effects of chronic anoxemia, chronic acidosis, and chronic hypercapnia in conditioning the response of patients with advanced pulmonary emphysema, patients without pulmonary disease who exhibited such derangements have also been studied. For investigation of the effects of chronic anoxemia, patients with cyanotic congenital heart disease were chosen; for the effects of chronic acidosis patients with long standing renal failure and nitrogen retention; and for the effects of hypercapnia patients with chronic metabolic alkalosis secondary to hyperadrenalism.

MATERIALS AND METHODS

Material for study comprised twelve normal subjects, thirteen patients with chronic pulmonary emphysema, three patients with cyanotic congenital heart disease, three patients with chronic renal disease and uremia, and two patients with Cushing's syndrome and chronic metabolic alkalosis.

Subjects were studied in the post-absorptive state. Observations at rest and during a standard exercise test were carried out as outlined by Baldwin, Cournand, and Richards (6). To determine sensitivity to the carbon dioxide-hydrogen ion stimulus, three sets of observations were made on each subject, allowing the determination of three points on the stimulus response curve. In each instance the subject breathed in succession room air, 3 per cent CO_2 in air, and 5 per cent CO_2 in air through a two-way low resistance respiratory valve with a dead space of 60 ml. The CO_2 mixture was supplied continuously from a tank through an anesthesia bag which was in turn connected to the inspiratory side of the respiratory valve. As previously reported by Nielsen (7), it was found that for both normal subjects and patients approximately twenty to twenty-five minutes' inhalation of the CO_2 gas mixtures used was required to achieve a steady state in terms of pulmonary ventilation,

[1] This work was done partly during the tenure of a Research Fellowship of the American Heart Association.

[2] Present Address: Department of Medicine, Baylor University College of Medicine, Houston, Texas.

[3] Supported in part by a gift from the Charles A. Frueauff Foundation.

† Dr. West died June 29, 1954.

respiratory frequency, and respiratory exchange ratio. Therefore observations were made only after twenty to thirty-five minutes of continuous inhalation of CO_2. Expired air was collected for a three-minute period, and a steadily and continuously drawn sample of arterial blood was obtained during the middle minute from an indwelling needle in a brachial artery.

If it is assumed that in a steady state there is a consistent relationship between the arterial blood concentrations and those intra- or extracellular concentrations of certain chemical agents acting at receptor and integrative levels on the nervous system, simultaneous measurement of blood concentrations and pulmonary ventilation permits estimation of the sensitivity of the respiratory nervous regulatory mechanism as a whole to these stimuli. The values relating effective alveolar ventilation, either in terms of ventilation ratio (VR) or alveolar ventilation per square meter body surface area ($\dot{V}A/M^2$), to changes in arterial CO_2 tension ($PaCO_2$) and hydrogen ion concentration $(H^+)a$ were obtained from graphs in which VR and $\dot{V}A/M^2$ were plotted against $PaCO_2$ and $(H^+)a$, respectively. For both $PaCO_2$ and $(H^+)a$ the relationship was found to be essentially linear, so that a straight line best fitting the three points could be drawn. The slopes of the lines so constructed have been utilized to determine the changes in arterial CO_2 tension or hydrogen ion concentration required to double the effective alveolar ventilation ($PaCO_2/VR$, $(H^+)a/VR$) and to determine the increase in effective alveolar ventilation per square meter body surface area associated with unit rise in arterial CO_2 tension or hydrogen ion concentration ($\dot{V}A/M^2/PaCO_2$, $\dot{V}A/M^2/(H^+)a$). To arrive at a figure representing the change in arterial CO_2 tension or hydrogen ion concentration necessary to double the effective alveolar ventilation when the latter did not actually occur, the observed slope was extrapolated.

Oxygen consumption and respiratory exchange ratios were calculated in the usual way from expired air analyses. Physiological dead space ventilation was obtained using the Bohr relationship for carbon dioxide (8) assuming the arterial CO_2 tension to be equivalent to the mean alveolar CO_2 tension (9, 10). Effective alveolar ventilation was taken as the difference between total pulmonary ventilation and dead space ventilation. Mean alveolar oxygen tensions were calculated from the usual "alveolar" equation when the inspired gas mixture was room air, and from a generalized form of the same relationships when carbon dioxide was added to the inspired air.[4] Arterial whole blood buffer base was calculated from the Singer-Hastings nomogram (13). A list of the symbols used (14) in this report appears in Table I.

TABLE I

Symbols

B.S.A.	Body Surface Area, square meters.
R_E	Respiratory exchange ratio (RQ), expired air.
R_A	Respiratory exchange ratio (RQ), alveolar air.
$\dot{V}O_2$	Oxygen consumption, ml. per min. STPD (Standard temperature and pressure, dry).
M^2	Square meters of body surface area.
$\dot{V}_E$	Total ventilation (expired air), liters per minute, BTPS (Body temperature and pressure, saturated with water vapor).
V_D	Ventilatory dead space total, *i.e.*, instrumental plus personal, ml. BTPS.
PAO_2	"Effective" alveolar oxygen tension, mm. Hg
$\dot{V}_A$	Effective alveolar ventilation, liters per min. BTPS.
VR	Alveolar ventilation ratio, *i.e.*, observed effective alveolar ventilation divided by resting effective alveolar ventilation.
pHs	Arterial blood pH (serum).
$(H^+)a$	Hydrogen ion concentration, arterial blood, in billionths of moles per liter.
$CsCO_2$	Serum carbon dioxide content, arterial blood, volumes per cent.
$PaCO_2$	Carbon dioxide tension, arterial blood, mm. Hg
SaO_2	Oxygen saturation, arterial blood, per cent.
Vc	Hematocrit, arterial blood.
$(B_{B^+})b$	Buffer base, whole blood, mEq. per liter.
f	Respiratory frequency as breaths per minute.
VC	Vital capacity, ml. BTPS.
V_T	Tidal volume, ml. BTPS.
FRC	Functional residual capacity, liters BTPS.
$\frac{RV}{TC}$	Residual volume to total capacity ratio.
MBC	Maximum breathing capacity, liters per min. BTPS.
$\frac{PaCO_2}{VR}$	Change in arterial CO_2 tension, mm. Hg, necessary to double the effective alveolar ventilation.
$\frac{\dot{V}_A/M^2}{PaCO_2}$	Increase in effective alveolar ventilation, liters per minute BTPS per sq. meter BSA, associated with a rise of 1 mm. Hg in arterial CO_2 tension.
$\frac{(H^+)a}{VR}$	Change in arterial hydrogen ion concentration, billionths of moles per liter, necessary to double the effective alveolar ventilation.
$\frac{\dot{V}_A/M^2}{(H^+)a}$	Increase in effective alveolar ventilation, liters per minute BTPS per sq. meter BSA, associated with a rise in arterial hydrogen ion concentration of 1 billionth of a mole per liter.

Total pulmonary ventilation per unit time and respiratory frequency were measured with a Tissot gasometer and suitable recording apparatus. Fractions of CO_2 and

[4] The general form of the alveolar equation may be readily derived by expressing the alveolar respiratory exchange ratio in terms of fractions of oxygen and CO_2 in alveolar and inspired air, converting certain of these dry fractions to gas tension, and solving the expression for alveolar oxygen tension (11, 12). The form used in the present study was as follows:

$$P_{AO_2} = \frac{(P_B-47)(R_A \cdot F_{IO_2}+F_{ICO_2})+P_{ACO_2}[1-F_{ICO_2}(1-R_A)]}{R_A+F_{ICO_2}(1-R_A)}$$

This reduces to the usual alveolar equation in the special case where CO_2 in the inspired air is negligible. R_A was taken as equivalent to R_E.

oxygen in inspired and expired air were determined by the Scholander technique (15). Arterial blood oxygen content, CO_2 content, and oxygen capacity were determined on the Van Slyke manometric apparatus. Arterial blood pH was measured with a Cambridge glass electrode pH meter at 38° C. within ten minutes of removal from the vessel, transfer of blood from artery to glass electrode being accomplished without exposure to air. Arterial serum CO_2 content was calculated from the diagram of Van Slyke and CO_2 tension from the Henderson-Hasselbalch relationship. Lung volumes and maximum breathing capacity were measured with a Benedict-Roth respirometer. The open circuit method was used for determining functional residual capacity (16).

RESULTS

Normal group

The data obtained from observations on twelve normal subjects appear in Tables II–V, with the spirometric and ventilatory data in Tables II and III. Respiratory exchange ratios showed only small variation, there being an average change of 0.06 from the resting value with inhalation of 3 per cent CO_2 and 0.05 with 5 per cent CO_2. Respiratory frequency tended to increase only slightly with CO_2 inhalation, usually not more than about five breaths per minute. In two instances the rate was slower with 5 per cent CO_2 inhalation than with 3 per cent CO_2. A greater tidal volume largely accounted for the increased pulmonary ventilation with CO_2 inhalation, rising to 158 per cent of the resting tidal volume on 3 per cent CO_2, and to 232 per cent on 5 per cent CO_2. Oxygen consumption per square meter body surface area showed no consistent or marked change with 3 per cent CO_2 inhalation, and only an inconstant tendency to rise with 5 per cent CO_2 (average increase 11 ml.). Average total pulmonary ventilation rose to 202 per cent of the resting value with 3 per cent CO_2 inhalation, and to 329 per cent with 5 per cent CO_2. In all instances but two, the physiological dead space increased appreciably with 5 per cent CO_2 inhalation. If the three instances in which a large dead space flutter valve was used are excluded, and the personal dead space is determined by deducting the instrumental dead space (60 ml.) from the observed total dead space (V_D), then there was an average increase in the personal physiological dead space of 39 ml. or 47 per cent with 3 per cent CO_2 inhalation, and of 75 ml. or 61 per cent with 5 per cent CO_2. The average personal physiological dead space was 92 ml. The average values for calculated mean alveolar oxygen tension were 97 mm. Hg at rest, 117 mm. Hg with 3 per cent CO_2 inhalation, and 125 mm. Hg with 5 per cent CO_2. An increase of 3 per cent and 5 per cent CO_2 occurred in each instance, with little individual variation from the average figures. Average effective alveolar ventilation rose to 222 per cent of the resting value with 3 per cent CO_2 inhalation, and to 382 per cent with 5 per cent CO_2.

Table IV contains the data from arterial blood analyses. The pH values are also expressed in terms of absolute hydrogen ion concentration as billionths of moles per liter [6] in order that the

[6] Hydrogen ion concentration as billionths of moles per liter is related to pH in the following way: $(H^+) = \text{antilog } (9\text{-pH})$.

TABLE II

Spirometric data in twelve normal subjects *

Subject	Sex	Age	B.S.A.	Vital capacity		Maximum breathing capacity		
				ml.	*% pred.*	*L./min.*	*L./min./M²*	*% pred.*
T. J.	F	43	1.99	3,340	100	78	39	78
J. R. W.	M	35	1.91	4,200	100	144	75.4	100
J. A. W.	M	30	1.96	5,380	100	174	89	100
J. K. A.	M	32	2.10	7,050	100	182	87	100
H. J.	M	17	1.89	4,670	100	106	56	74
J. D.	M	37	1.73	4,685	100	107	62	93
H. M.	M	35	1.79	5,030	100	120	67	100
P. P.	M	35	1.68	4,130	100	121	71.9	100
V. M.	M	18	1.67	4,360	100	137	82.2	100
N. B.	F	27	1.70	3,610	100	151	89	100
B. O.	M	32	1.76	4,530	100	122	69.5	100
M. B.	F	24	1.52	3,050	100	108	72	100

* A value of 100 for per cent predicted vital or maximum breathing capacity indicates that the observed value was 100 per cent or above.

TABLE III

Physiologic data relative to pulmonary ventilation at rest and during CO_2 inhalation in twelve normal subjects

Subject	Inspired gas mixture	Time on mixture (*min.*)	f	R_E	$\dot{V}_{O_2}/M^3$	V_T	$\dot{V}_E$	V_D *ml.*	V_D % $\dot{V}_E$	Calc. $P_{A_{O_2}}$
T. J.	Room air	—	17.7	0.70	125	381	6.76	163	42.8	96
	3% CO_2	20	31	0.72	147	487	15.10	183	37.7	117
	5% CO_2	24	33.3	0.72	165	891	29.65	321	36	128
J. R. W.*	Room air	—	8.7	0.77	123	592	5.16	192	32.3	94
	3% CO_2	26	12.4	0.87	130	982	12.16	382	38.9	117
	5% CO_2	21	14	0.82	133	1,377	19.26	520	37.7	125
J. A. W.*	Room air	—	9	0.75	127	595	5.36	218	36.7	96
	3% CO_2	27	12	0.76	117	838	10.07	311	37.1	116
	5% CO_2	30	13.7	0.76	149	1,420	19.48	408	28.7	126
J. K. A.	Room air	—	11	0.73	96	433	4.76	149	34.3	96
	3% CO_2	26	11	0.73	122	836	9.20	170	20.3	115
	5% CO_2	32	13.7	0.77	149	1,274	17.43	161	12.6	124
H. J.*	Room air	—	18	0.86	137	425	7.64	170	39.9	100
	3% CO_2	29	21.3	0.83	139	600	12.80	175	29.2	117
	5% CO_2	29	22.3	0.76	125	912	20.35	380	41.7	124
J. D.	Room air	—	15.3	0.74	140	422	6.44	185	43.8	91
	3% CO_2	28	17	0.74	118	586	9.96	237	40.6	111
	5% CO_2	28	19	0.84	125	729	13.87	148	20.3	122
H. M.	Room air	—	24.3	0.83	120	296	7.09	149	50.6	92
	3% CO_2	26	25	0.96	108	504	12.60	217	43.1	115
	5% CO_2	28	27.7	0.84	115	658	18.24	300	45.6	120
P. P.	Room air	—	19.7	0.83	107	282	5.53	129	45.8	91
	3% CO_2	26	25.7	0.95	112	471	12.10	195	41.5	118
	5% CO_2	26	26.3	0.74	123	593	15.60	227	38.4	122
V. M.	Room air	—	9.3	0.77	96	424	3.94	133	31.3	97
	3% CO_2	25	13.7	0.86	141	959	13.14	246	25.7	120
	5% CO_2	27	17.7	0.77	121	1,193	21.13	382	32.1	127
N. B.	Room air	—	15.3	0.86	110	419	6.42	164	39.2	105
	3% CO_2	30	24	0.96	105	549	13.17	171	31.2	124
	5% CO_2	26	21.3	0.85	116	922	19.68	201	21.8	128
B. O.	Room air	—	15	0.83	115	405	6.07	166	41	101
	3% CO_2	30	17.3	0.88	118	626	10.83	162	26	120
	5% CO_2	31	18.3	0.77	114	948	17.35	116	12	128
M. B.	Room air	—	20.7	0.77	116	272	5.62	128	47.2	99
	3% CO_2	33	23	0.80	109	376	8.64	136	36.1	117
	5% CO_2	37	22.3	0.70	111	632	14.10	181	28.7	126

* Large dead space flutter valve used.

changes in ventilation may be related to these concentrations after the manner of Gray (17). Average values for arterial blood pH were 7.42 at rest, 7.41 with 3 per cent CO_2 inhalation, and 7.38 with 5 per cent CO_2. Hydrogen ion concentrations expressed as billionths of moles per liter corresponding to the above pH values are 37.9, 38.8, and 41.7, respectively. Average values for arterial CO_2 tension in mm. Hg were 41.5 at rest, 44 with 3 per cent CO_2 inhalation, and 46.5 with 5 per cent CO_2. Although tending to rise with CO_2 inhalation, the changes in oxygen saturation of the arterial blood were somewhat less consistent, presumably because of inability to measure accurately with the Van Slyke technique the small changes in oxygen saturation associated with increased oxygen tension in this region of the dissociation curve. Arterial hematocrit showed no appreciable change. No direct observations on urinary constituents were made to evaluate possible renal adjustments to the respiratory acidosis induced during the thirty-minute period of CO_2

breathing. However, the absence of any appreciable change in the whole blood buffer base at the end of the CO_2 inhalation period suggests that such adjustments as may have occurred were not significant.

Observations on changes in effective alveolar ventilation, and correlation of these changes with hydrogen ion concentration and CO_2 tension in the arterial blood are described in Table V. If the effective alveolar ventilation is measured in terms of liters per minute per square meter body surface area, the average value at rest is 1.93. Body surface area has been arbitrarily chosen here as a reference point which would permit some comparison of alveolar ventilation, oxygen consumption, and sensitivity to chemical respiratory stimuli among the normal subjects and the other groups studied. The age range in this group of normal subjects was 17 to 43 years, average age 30.4. Robinson's data (18) for the closest average age group (35.1 years) yield a figure of 2.07 liters per minute per square meter body surface area for effective alveolar ventilation. Typical stimulus-response curves or sensitivity slopes of a

TABLE IV

Physiologic data relative to the arterial blood at rest and during CO_2 inhalation in twelve normal subjects

Subject	Inspired gas mixture	pHs	$(H^+)a$	Cs_{CO_2}	Pa_{CO_2}	Sa_{O_2}	Vo	Calc. $(B_{B^+})b$
T. J.	Room air	7.42	38.0	58.1	39	92	33	47
	3% CO_2	7.41	38.9	58.6	40.5	97	33	47
	5% CO_2	7.37	42.6	59.2	45	95	34	47
J. R. W.	Room air	7.42	38.0	66.3	45	96	41	51
	3% CO_2	7.41	38.9	67.0	46.5	96	42	51
	5% CO_2	7.39	40.7	67.8	49.5	100	41	51
J. A. W.	Room air	7.40	39.8	61.4	43	98	42	49
	3% CO_2	7.39	40.7	61.8	45	98	42	49
	5% CO_2	7.37	42.7	63.5	48	99	43	49
J. K. A.	Room air	7.45	35.5	64.1	40.5	97	37	50
	3% CO_2	7.42	38.0	64.6	43	100	37	50
	5% CO_2	7.40	39.8	66.3	47.5	100	39	50
H. J.	Room air	7.42	38.0	63.3	42.5	99	40	50
	3% CO_2	7.41	38.9	63.5	43.5	98	40	50
	5% CO_2	7.40	39.8	64.1	45	97	41	50
J. D.	Room air	7.44	36.3	67.3	43	97	41	52
	3% CO_2	7.42	38.0	66.1	44.5	99	41	51
	5% CO_2	7.40	39.8	66.4	47	96	42	51
H. M.	Room air	7.40	39.8	62.7	44.5	96	41	49
	3% CO_2	7.39	40.7	62.7	46	96	42	50
	5% CO_2	7.37	42.7	63.6	48	96	42	49
P. P.	Room air	7.42	38.0	64.9	43.5	95	39	50
	3% CO_2	7.41	38.9	66.4	45.5	95	39	51
	5% CO_2	7.39	40.7	64.7	47	97	40	49
V. M.	Room air	7.40	39.8	56.1	39.5	97	42	47
	3% CO_2	7.39	40.7	57.7	41.5	98	43	47
	5% CO_2	7.38	41.7	57.1	42.5	99	43	46
N. B.	Room air	7.43	37.2	55.4	37	98	37	47
	3% CO_2	7.40	39.8	56.6	40	99	38	47
	5% CO_2	7.36	43.7	59.6	46	99	38	47
B. O.	Room air	7.44	36.3	60.3	40.5	98	47	51
	3% CO_2	7.42	38.0	65.1	43.5	96	47	51
	5% CO_2	7.40	39.8	64.9	45.5	97	48	51
M. B.	Room air	7.43	37.2	59.6	40	99	37	49
	3% CO_2	7.39	40.7	61.6	44.5	99	36	48
	5% CO_2	7.35	44.7	60.6	47	96	36	47

TABLE V

Physiologic data relative to effective alveolar ventilation and sensitivity to the carbon dioxide-hydrogen ion stimulus in twelve normal subjects

Subject	Inspired gas mixture	$\dot{V}_A$	$\dot{V}_A/M^2$	VR	$\frac{Pa_{CO_2}}{VR}$	$\frac{\dot{V}_A/M^2}{Pa_{CO_2}}$	$\frac{(H^+)a}{VR}$	$\frac{\dot{V}_A/M^2}{(H^+)a}$
T. J.	Room air	3.86	1.94	1				
	3% CO_2	9.40	4.72	2.33	1.5	1.4	1.2	1.8
	5% CO_2	19.	9.55	4.92				
J. R. W.	Room air	3.48	1.82	1				
	3% CO_2	7.43	3.89	2.13	1.7	1.2	1.1	1.9
	5% CO_2	12.	6.28	3.44				
J. A. W.	Room air	3.38	1.72	1				
	3% CO_2	6.33	3.23	1.87	1.6	1.1	0.9	1.9
	5% CO_2	13.88	7.08	4.12				
J. K. A.	Room air	3.13	1.49	1				
	3% CO_2	7.33	3.49	2.33	1.8	0.9	1.2	1.5
	5% CO_2	15.25	7.26	4.87				
H. J.	Room air	4.58	2.42	1				
	3% CO_2	9.05	4.79	1.97	1.5	1.6	1.1	2.2
	5% CO_2	11.83	6.26	2.58				
J. D.	Room air	3.62	2.09	1				
	3% CO_2	5.91	3.42	1.63	2.0	1.0	1.7	1.1
	5% CO_2	11.10	6.42	3.06				
H. M.	Room air	3.50	1.96	1				
	3% CO_2	7.17	4.01	2.05	1.9	1.1	1.5	1.4
	5% CO_2	9.92	5.54	2.84				
P. P.	Room air	3.	1.79	1				
	3% CO_2	7.07	4.21	2.36	1.5	1.2	1.1	1.7
	5% CO_2	9.60	5.71	3.19				
V. M.	Room air	2.71	1.62	1				
	3% CO_2	9.75	5.84	3.60	0.8	2.3	0.5	3.8
	5% CO_2	14.30	8.56	5.27				
N. B.	Room air	3.90	2.30	1				
	3% CO_2	9.06	5.33	2.32	2.7	0.9	2.0	1.1
	5% CO_2	15.40	9.06	3.95				
B. O.	Room air	3.58	2.03	1				
	3% CO_2	8.02	4.56	2.24	1.5	1.3	0.9	2.2
	5% CO_2	15.23	8.65	4.25				
M. B.	Room air	2.97	1.95	1				
	3% CO_2	5.53	3.64	1.86	2.2	0.8	2.9	0.7
	5% CO_2	10.04	6.60	3.38				
				Average:	1.7	1.2	1.3	1.8
				Range	0.8–2.7	0.8–2.3	0.5–2.9	0.7–3.8
				S.D.	±0.5	±0.3	±0.6	±0.7

normal subject, constructed in the manner previously indicated, are shown in Figures 1 and 2.

To double the effective alveolar ventilation at rest an average increase of 1.7 mm. Hg (range 0.8 to 2.7, S.D. ± 0.5) in arterial CO_2 tension was required, associated with an average increase in arterial hydrogen ion concentration of 1.3×10^{-7} moles per liter (range 0.5 to 2.9, S.D. ± 0.6). For each mm. Hg rise in CO_2 tension of the arterial blood there was an average increase in effective alveolar ventilation per square meter body surface area of 1.2 liters per minute (range 0.8 to 2.3, S.D. ± 0.3). For an increase of 1.0×10^{-7} moles per liter in hydrogen ion concentration of the arterial blood an average increase in effective alveolar ventilation per square meter body surface

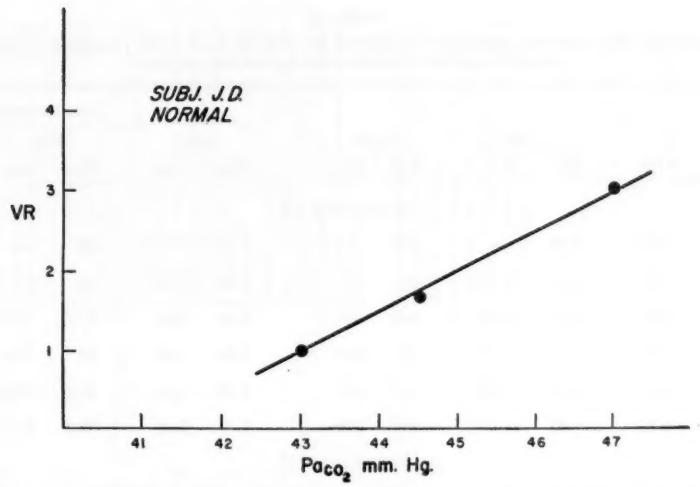

FIG. 1. STIMULUS RESPONSE CURVE IN A NORMAL SUBJECT, RELATING ARTERIAL CO_2 TENSION TO ALVEOLAR VENTILATION RATIO

area of 1.9 liters per minute (range 0.7 to 3.8, S.D. ± 0.7) occurred.

Pulmonary emphysema

Thirteen patients with chronic pulmonary emphysema were studied. All tolerated the CO_2 inhalation well, without respiratory distress. A few complained of mild headache following the procedure. The patients have been separated into three groups according to the classification of Baldwin, Cournand, and Richards (1). Those in Group II were found to have arterial oxygen un-

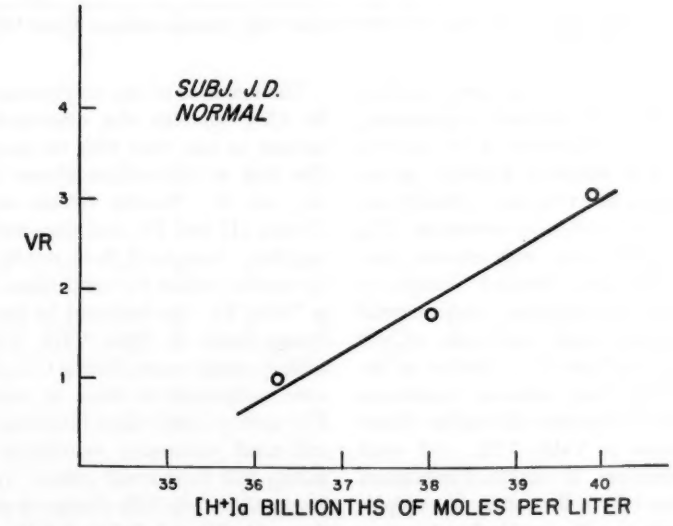

FIG. 2. STIMULUS RESPONSE CURVE IN A NORMAL SUBJECT, RELATING ARTERIAL HYDROGEN ION CONCENTRATION TO ALVEOLAR VENTILATION RATIO

TABLE VI

Physiologic data relative to pulmonary ventilation and arterial blood under conditions of rest and standard exercise in thirteen emphysematous subjects *

Subject				$\dot{V}_E/M^2$		$\dot{V}_{O_2}/M^2$		Arterial blood					
								pHs		Sa_{O_2}		Pa_{CO_2} mm. Hg	
Sex		Age	B.S.A.	Rest	Exer.	Rest	Exer.	Rest	Exer.	Rest	Exer.	Rest	Exer.
						Emphysema IV							
F	A. M.	47	1.65	4.30	—	137	—	7.33	—	59	—	54	—
M	F. J.	47	1.75	3.45	11.15	134	—	7.38	7.28	78	75	54	64
M	W. D.	58	1.64	5.00	12.60	160	306	7.46	7.42	87.6	54.8	45	50
M	R. C.	74	1.81	4.79	7.91	141	242	7.44	7.43	86	75.8	49.5	51.5
M	J. R.	52	1.79	3.74	5.88	139	207	7.38	7.32	81.6	53.8	59	66
M	C. M.	70	1.53	5.95	12.67	145	306	7.36	7.36	88.1	84.7	55	52
						Emphysema III							
M	G. R.	56	1.82	5.05	16.9	141	575	7.40	7.39	97.6	93	48	49
M	R. D.	75	1.54	3.99	10.15	119	301	7.39	7.36	90.4	86.4	48	53
M	J. Y.	46	1.83	4.83	12.60	143	405	7.40	7.29	80.6	67.7	47	60
						Emphysema II							
M	D. C.	48	1.61	5.70	12.61	127	374	7.43	7.42	93	90	40	40
M	G. B.	69	1.46	7.36	19.60	166	486	7.40	7.40	89	89	41	37
F	R. F.	54	1.60	4.69	15.12	134	436	7.40	7.38	96.2	90.5	36	36.5
M	A. H.	48	1.89	5.63	14.47	133	402	7.44	7.45	92.2	92.7	36	35

* In some instances these studies were done on a different day than those recorded in Tables VIII and IX, so that resting values are not exactly the same.

saturation at rest or following standard exercise, but no CO_2 retention. In addition to anoxemia, those in Group III had elevation of the arterial CO_2 tension at rest or following exercise, but no evidence of congestive heart failure. Finally, patients in Group IV suffered from anoxemia, CO_2 retention and cor pulmonale, with chronic congestive failure. The data obtained relative to ventilation, oxygen consumption, and arterial blood in these patients under conditions of rest and exercise appear in Table VI. Indices of intrapulmonary mixing, lung volumes, maximum breathing capacities, and serum electrolytes where available, are shown in Table VII. All were found to have reductions in vital and maximum breathing capacities. Both the index of intrapulmonary gas mixing and the residual volume to total capacity ratio were increased in all instances except one.

The response of the emphysematous subjects to the CO_2 stimulus was determined in the same manner as was done with the normal individuals. The data so derived are shown in Tables VIII, IX, and X. Similar results were obtained in Groups III and IV, and they may be considered together. Certain of these results, with the average normal values for comparison, are summarized in Table XI. As indicated by the respiratory exchange ratios in Table VIII, it was possible to achieve steady states during CO_2 inhalation which were comparable to those in normal individuals. The average percentage increases in tidal volume and total pulmonary ventilation were approximately half the normal values. As in the normal group, there was little change in oxygen consumption with CO_2 inhalation, but the average oxygen consumption of 137 ml. per square meter body surface area at rest for the emphysematous pa-

TABLE VII

Physiologic data relative to intrapulmonary gas mixing, lung volumes, and maximum breathing capacity with clinical findings in thirteen emphysematous subjects

Subject	Index of intrapulmonary mixing RV/TC	Vital capacity ml.	Vital capacity % pred.	Total capacity ml.	Total capacity % pred.	Max. breathing cap. L./min.	Max. breathing cap. L./min./M²	Max. breathing cap. % pred.	Serum electrolytes mEq./L. Na	K	Cl	BUN mg. %	Notes
						Emphysema IV							
A. M.	—	—	1,570	59	—	—	17.8	10.8	22	142.3	5.1	101.4	14	Marked congestive failure. Could not be exercised. FRC not done. Marked exp. prolongation. VC & MBC increased p bronchodilator.*
F. J.	3.33	46	2,230	60	4,160	86	27.9	16	26	—	—	—	15	Chronic congestive failure. Marked exp. prolongation. VC & MBC increased p bronchodilator.
W. D.	5.98	72	1,520	42	5,540	106	14.1	8.6	14	145	5.0	95	(NPN 38)	Mild congestive failure. Exp. prolongation. VC & MBC increased p bronchodilator.
R. C.	11.56	57	1,784	54	4,142	87	15.4	8.5	18	—	—	98.6	19	Exercise 18 steps only in one min. Mild congestive failure. Exp. prolongation. VC & MBC increased p bronchodilator.
J. R.	5.82	50	2,470	65	4,962	90	22.8	12.9	22	—	—	—	—	Exercise 25 steps in one min. Chronic congestive failure, mild. Exp. prolongation. Little improvement p bronchodilator.
C. M.	5.99	50	1,898	59	3,823	82	48.7	32	64	143.6	5.0	94.0	33	Mild congestive failure. Exp. prolongation. MBC increased p bronchodilator.
						Emphysema III							
G. R.	3.49	48	2,540	68	4,163	77	92.2	50.6	88	—	—	—	16.5	Diffuse infiltration both lungs by x-ray consistent with clinical diagnosis of asbestosis.
R. D.	6.93	59	1,992	67	4,815	112	27.5	17.8	40	—	—	—	18	Exercise 26 steps in one min. Exp. prolongation. VC & MBC increased p bronchodilator.
J. Y.	9.93	58	2,755	68	5,290	125	49.5	27	43	—	—	—	—	Modest increases in VC & MBC p bronchodilator.
						Emphysema II							
D. C.	3.42	30	3,340	89	4,785	98	54.1	33.6	55	—	—	—	—	Expiratory prolongation. MBC increased p bronchodilator.
G. B.	5.15	54	2,860	75	6,220	110	52.2	37.8	75	136.6	5.0	98	23	Exercise 25 steps in one min. Exp. prolongation. Little improvement p bronchodilator.
R. F.	2.06	41	1,520	57	2,597	68	28.2	17.6	44	—	—	—	23	Bronchiectasis. Slight exp. prolongation. VC and MBC increased p bronchodilator.
A. H.	4.07	54	1,860	46	5,190	78	43.9	23.5	38	135	4.5	95.6	10	Congestive circulatory failure secondary to constrictive pericarditis, the latter suspected on basis of cardiac catheterization data, confirmed by pericardial biopsy. Marked improvement in VC and MBC p bronchodilator.

* Bronchodilator agent used in these studies was "Vaponefrin," a 2.25 per cent solution of racemic epinephrine hydrochloride.

TABLE VIII

Physiologic data relative to pulmonary ventilation at rest and during CO_2 inhalation in thirteen emphysematous subjects

Subject	Inspired gas mixture	Time on mixture (*min.*)	f	R_E	$\dot{V}_{O_2}/M^2$	V_T	$\dot{V}_E$	V_D *ml.*	V_D % $\dot{V}_E$	Calc. $P_{A_{O_2}}$
					Emphysema IV					
A. M.	Room air	—	21.3	0.73	137	332	7.10	207	62.3	80
	3% CO_2	30 min.	24	0.70	145	392	9.41	226	57.6	97
	5% CO_2	24 min.	24	0.67	178	563	13.50	322	57.2	103
F. J.	Room air	—	15.7	0.70	131	366	5.74	163	44.6	88
	3% CO_2	28 min.	16.3	0.83	118	610	9.95	302	49.5	107
	4% CO_2	33 min.	17.8	0.65	143	656	11.68	328	50.2	109
W. D.	Room air	—	17	0.76	160	483	8.20	253	52.3	91
	3% CO_2	26 min.	17.2	0.80	153	683	11.74	317	46.3	107
	4% CO_2	26 min.	21.2	0.76	179	691	14.62	306	44.2	111
R. C.	Room air	—	23.7	0.73	141	366	8.67	227	62.1	84
	3% CO_2	30 min.	24.7	0.73	137	483	11.92	293	60.6	98
	5% CO_2	25 min.	26.7	0.64	163	572	15.28	349	61.0	100
J. R.	Room air	—	13.7	0.79	139	483	6.62	275	57.0	77
	3% CO_2	32 min.	15	0.81	130	543	8.15	288	53.0	93
	5% CO_2	30 min.	15.7	0.84	125	655	10.28	344	52.5	102
C. M.	Room air	—	19.7	0.74	145	462	9.10	329	71.4	79
	3% CO_2	25 min.	25	0.77	128	487	12.17	353	72.7	95
	5% CO_2	25 min.	24	0.78	135	644	15.45	455	70.6	103
					Emphysema III					
G. R.	Room air	—	20.67	0.77	135	430	8.90	262	60.7	92
	3% CO_2	25 min.	20.34	0.79	127	653	13.25	387	59.3	111
	5% CO_2	22 min.	21	0.73	153	950	19.95	493	51.8	118
R. D.	Room air	—	13.7	0.82	119	448	6.14	250	55.8	91
	3% CO_2	30 min.	16.7	0.88	113	633	10.55	354	55.9	109
	5% CO_2	28 min.	20	0.70	123	758	15.17	463	61.2	113
J. Y.	Room air	—	20.3	0.85	128	458	9.30	286	62.4	89
	3% CO_2	26 min.	18.7	0.77	140	643	12.	347	54.0	105
	5% CO_2	25 min.	20.7	0.78	159	814	16.85	424	52.2	107
					Emphysema II					
D. C.	Room air	—	23.0	0.76	146	403	9.27	224	55.7	102
	3% CO_2	28 min.	24.7	0.74	159	696	17.20	272	39	110
	4% CO_2	28 min.	28.3	0.83	161	868	24.60	267	30.7	130
G. B.	Room air	—	23.7	0.80	166	453	10.73	281	61.8	99
	3% CO_2	28 min.	25.3	0.91	147	662	16.76	347	52.5	120
	5% CO_2	28 min.	26	0.84	178	975	25.30	396	40.7	125
R. F.	Room air	—	22.3	0.77	134	337	7.51	158	47.1	104
	3% CO_2	30 min.	27.7	0.75	130	513	14.23	202	39.3	124
	5% CO_2	28 min.	32	0.81	117	722	23.10	213	29.6	131
A. H.	Room air	—	16	0.75	132	519	8.30	764	50.9	98
	3% CO_2	29 min.	23.7	0.75	138	694	16.45	338	48.6	117
	5% CO_2	25 min.	25.3	0.78	162	1,054	26.68	431	40.8	123

tients was higher than the average figures for the normal group by 19 ml. Although the changes in physiological dead space with CO_2 inhalation were approximately the same as the normal percentagewise, the average personal dead space at rest of 208 ml. was twice the normal, and the absolute changes were almost twice as great. The alveolar oxygen tensions were consistently lower at rest than in the normal group, with a lesser degree of change during CO_2 inhalation.

The relationships between effective alveolar ventilation and arterial blood CO_2 tensions and hy-

drogen ion concentration are found in Table X. The data were handled in the same way as in the normal group, and the stimulus response relationships obtained were found to be linear. Although the age groups are not comparable, it may be noted that the average effective alveolar ventilation per square meter body surface area at rest (1.84 liters) in the Group III and IV emphysema categories was slightly lower than that of 1.93 liters for the normal group. Thus the elevated arterial CO_2 tension at rest in the emphysematous groups would presumably stem from the effects of

TABLE IX

Physiologic data relative to the arterial blood at rest and during CO_2 inhalation in thirteen emphysematous subjects

Subject	Inspired gas mixture	pHs	$(H^+)a$	Cs_{CO_2}	Pa_{CO_2}	Sa_{O_2}	Vc	Calc. $(B_{B^+})b$
			Emphysema IV					
A. M.	Room air	7.33	46.8	65.3	54	59	47	49
	3% CO_2	7.30	50.1	65.3	58	77	50	49
	5% CO_2	7.28	52.5	69.0	64	77	55	49
F. J.	Room air	7.41	38.9	64.0	44	79	48	51
	3% CO_2	7.35	44.7	65.4	52	87	48	50
	5% CO_2	7.33	46.8	65.2	54	87	49	49
W. D.	Room air	7.46	34.7	72.6	45	88	55	56
	3% CO_2	7.45	35.5	78.6	50	91	55	58
	5% CO_2	7.43	37.2	79.7	53	91	56	58
R. C.	Room air	7.44	36.6	76.0	49.5	86	41	55
	3% CO_2	7.39	41.0	78.9	57.5	92	42	55
	5% CO_2	7.35	44.9	80.5	64	92	45	55
J. R.	Room air	7.38	41.6	79.8	59	82	52	55
	3% CO_2	7.36	44.0	81.8	64	89	51	56
	5% CO_2	7.34	45.7	81.8	67	92	52	56
C. M.	Room air	7.36	43.7	71.4	55	88	60	52
	3% CO_2	7.32	47.8	72.0	61	94	60	52
	5% CO_2	7.30	50.1	73.5	65	95	59	52
			Emphysema III					
G. R.	Room air	7.42	38.0	69.0	47	88	41	53
	3% CO_2	7.39	40.7	68.4	50	95	41	52
	5% CO_2	7.37	42.7	70.2	53	—	42	51
R. D.	Room air	7.39	40.9	66.1	48	90	40	52
	3% CO_2	7.36	43.3	67.3	52	95	40	50
	5% CO_2	7.34	45.8	68.8	56	94	41	50
J. Y.	Room air	7.37	42.6	66.1	50	87	48	51
	3% CO_2	7.36	43.7	68.6	53	94	48	51
	5% CO_2	7.29	51.2	68.5	62	99	48	50
			Emphysema II					
D. C.	Room air	7.46	34.7	61.1	38	90	41	50
	3% CO_2	7.45	35.5	61.1	39	91	41	50
	5% CO_2	7.43	37.2	62.0	41	95	42	50
G. B.	Room air	7.40	39.8	58.0	41	89	46	48
	3% CO_2	7.39	40.7	60.0	43	91	47	49
	5% CO_2	7.37	42.7	61.4	46	97	47	49
R. F.	Room air	7.40	40.1	50.3	36	96	39	45
	3% CO_2	7.39	40.9	51.8	37.5	96	40	45
	5% CO_2	7.33	46.6	51.8	42	95	42	44
A. H.	Room air	7.44	36.3	62.4	40	92	43	50
	3% CO_2	7.43	37.2	62.2	41.5	95	44	50
	5% CO_2	7.38	41.7	63.9	47	96	47	50

TABLE X

Physiologic data relative to effective alveolar ventilation and sensitivity to the carbon dioxide-hydrogen ion stimulus in thirteen emphysematous subjects

Subject	Inspired gas mixture	$\dot{V}_A$	$\dot{V}_A/M^2$	VR	$\frac{Pa_{CO_2}}{VR}$	$\frac{\dot{V}_A/M^2}{Pa_{CO_2}}$	$\frac{(H^+)a}{VR}$	$\frac{\dot{V}_A/M^2}{(H^+)a}$
				Emphysema IV				
A. R.	Room air	2.68	1.62	1				
	3% CO_2	3.99	2.42	1.49	8.6	0.2	5.7	0.3
	5% CO_2	5.78	3.50	2.15				
F. J.	Room air	3.18	1.82	1				
	3% CO_2	5.02	2.87	1.57	13.0	0.1	9.6	0.2
	5% CO_2	5.82	3.33	1.83				
W. D.	Room air	3.91	2.38	1				
	3% CO_2	6.32	3.85	1.61	7.4	0.3	2.3	1.1
	5% CO_2	8.16	4.98	2.08				
R. C.	Room air	3.29	1.82	1				
	3% CO_2	4.70	2.60	1.43	17.0	0.1	10.1	0.2
	5% CO_2	5.98	3.30	1.81				
J. R.	Room air	2.85	1.59	1				
	3% CO_2	3.83	2.14	1.34	12.0	0.1	6.7	0.3
	5% CO_2	4.88	2.72	1.71				
C. M.	Room air	2.60	1.70	1				
	3% CO_2	3.33	2.18	1.28	14.0	0.1	9.7	0.2
	5% CO_2	4.55	2.97	1.75				
				Emphysema III				
G. R.	Room air	3.48	1.91	1				
	3% CO_2	5.38	2.96	1.55	4.4	0.5	2.9	0.6
	5% CO_2	9.60	5.27	2.75				
R. D.	Room air	2.72	1.77	1				
	3% CO_2	4.66	3.03	1.71	6.5	0.3	4.0	0.5
	5% CO_2	5.90	3.83	2.17				
J. Y.	Room air	3.50	1.91	1				
	3% CO_2	5.52	3.02	1.58	8.4	0.2	5.0	0.4
	5% CO_2	8.04	4.39	2.30				
				Emphysema II				
D. C.	Room air	4.11	2.55	1				
	3% CO_2	10.33	6.42	2.52	0.9	2.9	0.8	3.4
	5% CO_2	17.02	10.57	4.14				
G. B.	Room air	4.10	2.81	1				
	3% CO_2	7.98	5.46	1.94	1.9	1.5	1.1	2.6
	5% CO_2	15.00	10.28	3.67				
R. F.	Room air	3.97	2.48	1				
	3% CO_2	8.66	5.41	2.18	1.7	1.3	1.5	1.6
	5% CO_2	16.25	10.17	4.10				
A. H.	Room air	4.07	2.15	1				
	3% CO_2	8.45	4.47	2.08	2.1	1.0	1.8	1.2
	5% CO_2	15.80	8.37	3.88				

diminished effective alveolar ventilation and some slight increase in CO_2 production, possibly related to greater respiratory work. The average percentage increases in effective alveolar ventilation with 3 per cent and 5 per cent CO_2 inhalation were less than half the average normal values. To double the effective alveolar ventilation, which was accomplished in only five of the nine cases, an increase of 4.4 to 17 mm. Hg in the arterial CO_2 tension was required. The changes were well out of the normal range in all instances, usually several times the average normal value. The as-

sociated change in hydrogen ion concentration necessary to double the effective alveolar ventilation was usually three or more times the average normal value, although in two instances it fell at the upper limit of the normal range. For each mm. Hg rise in the arterial CO_2 tension there was an increase in effective alveolar ventilation per square meter body surface area of only 0.1 to 0.5 liter per minute, as compared with an average increase of 1.2 liters per minute in the normal group. The smallest increase in the normal group was 0.8 liter per minute. For a billionth of a mole per liter increase in arterial blood hydrogen ion concentration, the effective alveolar ventilation per square meter body surface area rose only 0.2 to 1.1 liters per minute. The average value in the normal group was 1.9 liters per minute, and in only one instance was the value of a Group III or IV emphysema patient within the normal range.

Observations on four emphysematous patients without CO_2 retention (Group II) are shown in Tables VIII, IX and X. The findings at rest and changes with CO_2 inhalation are roughly comparable to the normal with regard to respiratory exchange ratio, respiratory frequency, oxygen consumption, tidal volume, total pulmonary ventilation, and calculated mean effective alveolar oxygen tension. The physiological dead space at rest is greater than normal as would be expected. Except for lowered oxygen saturation, the results of arterial blood analyses differ little from those described in the normal group. Effective alveolar ventilation per square meter body surface area at rest was greater than the average normal value in all instances. Nevertheless the changes in effective alveolar ventilation with CO_2 inhalation were essentially the same as in the normal group, *i.e.*, roughly two times the resting value with 3 per cent CO_2 inhalation, and four times the resting value with 5 per cent CO_2. Relationships between effective alveolar ventilation and arterial CO_2 tension or hydrogen ion concentration obtained in the manner previously described, fell within the normal range, indicating a normal ventilatory response to the carbon dioxide-hydrogen ion stimulus.

Cyanotic congenital heart disease

Of the patients with cyanotic congenital heart disease, the diagnosis of pulmonic stenosis and interatrial defect had been made in one, and that of Eisenmenger's Complex in two, on the basis of cardiac catheterization studies. Spirometric data and clinical notes on these patients are shown in Table XII. Vital and maximum breathing capacities were within the normal range except in one instance where moderate kyphosis probably accounted for some reduction in the vital capacity. Observations on ventilation and arterial blood at rest and during CO_2 inhalation appear in Tables XIII, XIV, and XV. All three patients had arterial oxygen unsaturation and polycythemia of appreciable degree. In addition two had lowered arterial CO_2 tensions and a tendency to alkalosis. Oxygen consumption per square meter body surface area, total pulmonary ventilation, and ef-

TABLE XI

Response of emphysematous subjects (groups III and IV) and normal subjects to CO_2 inhalation. Comparison of sensitivity to the carbon dioxide-hydrogen ion stimulus

Inspired gas mixture	pH average values		Pa_{CO_2} average increase		$P_{A_{O_2}}$ average values		$\dot{V}_E$ average increase		$\dot{V}_A$ average increase	
	Normal	Emphysema	Normal	Emphysema	Normal	Emphysema	Normal	Emphysema	Normal	Emphysema
			mm. Hg		*mm. Hg*		%		%	
Room air	7.42	7.40	—	—	97	86	—	—	—	—
3% CO_2 in air	7.41	7.36	2.5	5.1	117	102	202	144	222	151
5% CO_2 in air	7.38	7.34	5.0	9.6	125	107	329	192	382	206

	$\frac{Pa_{CO_2}}{VR}$	$\frac{\dot{V}_A/M^2}{Pa_{CO_2}}$	$(H^+)a/VR$	$\frac{\dot{V}_A/M^2}{(H^+)a}$
Normal average value	1.7	1.2	1.3	1.9
Emphysema range	4.4–17	0.1–0.5	2.3–10.1	0.2–1.1

TABLE XII

Spirometric data and clinical findings in patients with cyanotic congenital heart disease, chronic acidosis, and chronic alkalosis

Patient Sex	Age	B.S.A.	Vital capacity ml.	% pred.	Maximum breathing cap. L./min.	L./min./M²	% pred.	Notes
					Cyanotic congenital heart disease			
M	J. B. 23	1.67	4,630	108	124.5	75	100	Moderate reduction in exercise tolerance. Pulmonic stenosis with interatrial defect and right-to-left shunt.
M	R. W. 28	1.64	2,170	57	97.3	59.3	83	Kyphosis of moderate degree. Eisenmenger's Complex.
M	W. M. 26	1.86	5,880	137	170	91.3	126	Moderate reduction in exercise tolerance. Eisenmenger's Complex.
					Chronic acidosis			
F	G. K. 30	1.58	2,590	89	72	45.6	80	Chronic glomerulonephritis since age 5. Nitrogen retention documented for 3 yrs. prior to study. At time of study serum electrolyte levels, mEq./L., Na 135.8, Cl 98.6, K 4.7; BUN 83 mg. %.
M	J. K. 48	1.67	2,146	61	71.7	43	70	Nephritis since age 17 with documented nitrogen retention for 7 yrs. prior to study. Lowered venous serum CO_2 content found on occasion over several years. At time of study serum electrolyte levels, mEq./L., Na 131.4, Cl 104.6, K 5.6, BUN 96 mg. %.
M	E. W. 51	1.91	2,695	80	71.5	37.4	62	Documented nitrogen retention for 3 yrs. prior to study. Presumed diagnosis arteriolar nephrosclerosis. At time of study serum electrolyte levels, mEq./L., Na 139, Cl 125, K 7.1, BUN 58 mg. %.
					Chronic alkalosis			
F	K. S. 49	1.65	2,030	80	57.3	35	73	Functioning adrenal cortical carcinoma and secondary Cushing's syndrome. Documented hypochloremic alkalosis and CO_2 retention for two months prior to study. At time of study serum electrolytes, mEq./L., Na 138.2, Cl 96.2, K 3.9.
F	J. T. 41	1.59	4,330	152	153.4	96.5	187	Adrenal cortical hyperplasia with documented hypochloremic alkalosis for two months prior to therapy. No CO_2 retention for approx. 4 mos. at the time of study.

fective alveolar ventilation [6] at rest were uniformly elevated. The effective alveolar ventilation per square meter body surface area was 50 per cent or more greater than the average normal value in all three patients. With CO_2 inhalation, the changes

[6] The effective alveolar ventilation in these cases, as in the normal group, was calculated on the assumption that alveolar CO_2 tension was equivalent to arterial CO_2 tension. This of course is not a valid assumption in these patients because of venous admixture to left ventricular blood through the right to left intracardiac shunt. Thus the actual effective alveolar ventilation at rest was somewhat higher than that recorded in Table XV. Since no pulmonary arterial blood samples were obtained at the time of the CO_2 inhalation study which would allow estimation of the degree of venous admixture, no calculations were carried out attempting to correct arterial CO_2 tension to alveolar CO_2 tension. This would introduce no error in the slope of the CO_2 response curve if the degree of shunt remained constant with CO_2 inhalation. However, even if the amount shunted were to change by as much as 10 per cent with CO_2 inhalation, the difference in the corrected alveolar CO_2 tension would be no greater than 1 mm. Hg. Thus, no appreciable error has been introduced into the measurement of the *change* in effective alveolar ventilation with CO_2 inhalation, a partial determinant of the slope of the stimulus response curve. Because of the lack of specific values for alveolar CO_2 tension, no calculations of mean effective alveolar oxygen tension were carried out.

in respiratory exchange ratio and frequency, tidal volume, ventilation, physiological dead space, pH, CO_2 tension, and buffer base were similar to those observed in the normal group. However, the increase in oxygen consumption per square meter body surface area with 5 per cent CO_2 breathing in these patients was consistent, and was presumably related to the high rates of ventilation observed. As expected, only a small increase in arterial oxygen saturation occurred with CO_2 inhalation, despite substantial increments in effective alveolar ventilation. As in the normal group, the relationships between changes in effective alveolar ventilation and arterial CO_2 tension or hydrogen ion concentration were found to be essentially linear. The observed changes in arterial CO_2 tension and hydrogen ion concentration associated with doubling of the effective alveolar ventilation fell within the normal range, although an increased response in terms of effective alveolar ventilation per square meter of body surface area occurred in one instance.

Chronic metabolic acidosis

Three patients with uremia and chronic metabolic acidosis secondary to renal failure were studied. Clinical findings, blood electrolyte levels at the time of study, and spirometric data appear in Table XII.

All showed a tendency to reduction in maximum breathing capacity which might well have been related to the presence of weakness and easy fa-

TABLE XIII

Physiologic data relative to pulmonary ventilation at rest and during CO_2 inhalation in patients with cyanotic congenital heart disease, chronic acidosis, and chronic alkalosis

Patient	Inspired gas mixture	Time on mixture (*min.*)	f	R_E	$\dot{V}_{O_2}/M^2$	V_T	$\dot{V}_E$	V_D *ml.*	V_D % $\dot{V}_E$	Calc. $P_{A_{O_2}}$
					Cyanotic congenital heart disease *					
J. B.	Room air	—	18	0.76	148	524	9.43	212	40.3	
	3% CO_2	26	20.3	0.88	158	1,265	25.70	187	14.7	
	4% CO_2	30	25	0.71	206	1,750	43.70	120	7.0	
R. W.	Room air	—	31.7	0.76	179	388	12.30	228	58.7	
	3% CO_2	26	33.3	0.71	151	666	22.15	422	63.3	
	4% CO_2	29	36.7	0.74	200	904	33.20	477	52.8	
W. M.	Room air	—	16	0.75	162	680	10.88	271	39.8	
	3% CO_2	27	20.2	0.87	137	1,362	27.50	604	44.3	
	4% CO_2	26	23	0.71	203	2,010	46.15	888	44.1	
					Chronic acidosis					
G. K.	Room air	—	15.7	0.81	131	424	6.66	172	40.5	105
	3% CO_2	27	20	0.82	138	717	14.35	258	36.1	124
	5% CO_2	27	20.7	0.94	139	1,300	26.90	345	26.6	131
J. K.	Room air	—	29.7	0.80	187	387	11.48	211	54.5	101
	3% CO_2	23	29.3	0.72	232	773	22.65	359	46.4	119
	5% CO_2	Tolerated only 8 min.	49	—	—	992	48.60	—	—	—
W. E.	Room air	—	16.7	0.82	119	514	8.59	231	44.9	106
	3% CO_2	30	25.7	0.76	136	795	20.43	369	46.4	121
	5% CO_2	Tolerated only 10 min.	26	—	—	1,345	34.97	—	—	—
					Chronic alkalosis					
K. S.	Room air	—	20	0.73	148	320	6.41	139	43.3	93
	3% CO_2	34	23.7	0.72	136	435	10.33	182	41.8	114
	5% CO_2	30	23	0.81	137	693	15.93	267	38.5	120
J. T.	Room air	—	8.3	0.72	145	651	5.41	234	36.0	95
	3% CO_2	26	10	0.70	143	915	9.15	364	39.8	111
	5% CO_2	25	14	0.80	141	1,232	17.26	610	49.5	120

* See footnote 6.

TABLE XIV

Physiologic data relative to the arterial blood at rest and during CO_2 inhalation in patients with cyanotic congenital heart disease, chronic acidosis, and chronic alkalosis

Patient	Inspired gas mixture	pHa	(H⁺)a	Cs_{CO_2}	Pa_{CO_2}	Sa_{O_2}	Vc	Calc. (Bb_{+})b
			Cyanotic congenital heart disease					
J. B.	Room air	7.45	35.4	45.7	29	86	62	48
	3% CO_2	7.44	36.3	49.1	32	87	62	48
	4% CO_2	7.40	39.8	49.7	35	88	62	47
R. W.	Room air	7.40	39.8	53.7	38.5	83	75	50
	3% CO_2	7.39	40.7	56.0	41	85	76	49
	4% CO_2	7.36	43.6	55.9	43	88	77	48
W. M.	Room air	7.49	32.3	51.4	30	87	61	51
	3% CO_2	7.42	38.0	52.0	35	89	61	49
	4% CO_2	7.40	39.8	54.2	38.5	90	62	49
			Chronic acidosis					
G. K.	Room air	7.36	43.6	47.5	37	95	26	41
	3% CO_2	7.35	44.7	49.0	38.5	98	27	41
	5% CO_2	7.30	50.1	49.5	43	100	28	41
J. K.	Room air	7.32	47.4	50.1	41.5	94	33	42
	3% CO_2	7.32	48.3	49.3	42	96	35	42
	5% CO_2	—	—	—	—	—	—	—
W. E.	Room air	7.20	63.2	32.0	34.5	94	26	32
	3% CO_2	7.19	64.6	33.1	37	100	27	32
	5% CO_2	—	—	—	—	—	—	—
			Chronic alkalosis					
K. S.	Room air	7.47	33.9	69.2	42.5	91	38	54
	3% CO_2	7.45	35.5	70.5	45	96	38	54
	5% CO_2	7.42	38.0	73.2	50	96	37	54
J. T.	Room air	7.40	39.8	60.1	42	94	43	48
	3% CO_2	7.38	41.7	63.3	47	97	43	49
	5% CO_2	7.35	44.6	65.1	52	97	44	49

tigability. The lowered vital capacity in patient J. K. was possibly associated with the presence of moderate pulmonary congestion.

Observations on the response of these uremic patients to the CO_2 stimulus are found in Tables XIII, XIV, and XV. At rest all had an elevated effective alveolar ventilation per square meter body surface area, reduced arterial pH, and anemia. One patient had an elevated oxygen consumption per square meter body surface area. The other two had a somewhat lowered arterial CO_2 tension. Two of the three patients were not able to tolerate the 5 per cent CO_2 inspired gas mixture for longer than a few minutes, a finding confirmatory of Peabody's experience (19). In these two instances the ventilation measured near the end of this short period on 5 per cent CO_2 was 50 per cent or more of the maximum breathing capacity, suggesting that intolerance may result when the ventilatory drive exceeds the patient's physical capacity to respond. The sensitivity to the carbon dioxide-hydrogen ion stimulus in these two cases has been determined, therefore, on the basis of only two points on the stimulus response curve. There was a deficit in the calculated buffer base, as found by Yeomans and Stueck (20) in their uremic subjects, and no change in buffer base occurred with CO_2 inhalation.

It can be seen that the response to the carbon dioxide-hydrogen ion stimulus when evaluated in terms of either the alveolar ventilation ratio or effective alveolar ventilation per square meter body surface area fell within the normal range in two patients, but was distinctly increased in a third (J. K.).

Chronic metabolic alkalosis

Clinical notes on the two patients with chronic metabolic alkalosis and CO_2 retention secondary

to hyperadrenalism appear in Table XII. In both instances the anatomical diagnosis is based on gross and microscopic examination of the operative pathological specimen. It should be noted that there was no disturbance of blood acid-base balance in patient J. T. at the time of study. Although CO_2 retention was documented in this patient only for the two-month period of observation prior to therapy, clinical symptoms had been present for a year or more. The venous serum CO_2 content, previously about 35 mEq. per liter, fell to normal shortly after subtotal adrenalectomy, and remained so during the ensuing four months prior to study.

The spirometric observations on these two patients are shown in Table XII. The slight reductions in vital and maximum breathing capacities found in patient K. S. were probably the result of old pleural disease.

Tables XIII, XIV, and XV contain the data obtained during CO_2 inhalation in these two patients. The first was found to have an elevated arterial blood pH and serum bicarbonate level at rest, with a somewhat lowered oxygen saturation. Resting values for the second patient were within normal limits at the time of study. In both cases the ventilatory responses to 3 per cent and 5 per cent CO_2 inhalation were less than normal, and sensitivity to the carbon dioxide-hydrogen ion stimulus was one-half to one-third that represented by the average normal values.

DISCUSSION

The effects of CO_2 inhalation on the normal subjects were comparable to those found previously (21–25). In this study the average value of 1.7 mm. Hg change in arterial CO_2 tension required

TABLE XV

Physiologic data relative to effective alveolar ventilation and sensitivity to the carbon dioxide-hydrogen ion stimulus in patients with cyanotic congenital heart disease, chronic acidosis, and chronic alkalosis

Patient	Inspired gas mixture	$\dot{V}_A$	$\dot{V}_A/M^2$	VR	$\frac{Pa_{CO_2}}{VR}$	$\frac{\dot{V}_A/M^2}{Pa_{CO_2}}$	$\frac{(H^+)a}{VR}$	$\frac{\dot{V}_A/M^2}{(H^+)a}$
			Cyanotic congenital heart disease					
J. B.	Room air	5.63	3.37	1				
	3% CO_2	21.80	13.05	3.88	1.0	3.5	0.7	5.5
	4% CO_2	40.7	24.36	7.22				
R. W.	Room air	5.08	3.10	1				
	3% CO_2	8.12	4.95	1.60	2.2	1.5	1.8	1.7
	4% CO_2	15.68	9.56	3.08				
W. M.	Room air	6.54	3.53	1				
	3% CO_2	15.33	8.24	2.34	2.7	1.3	2.6	1.4
	4% CO_2	25.80	13.87	3.94				
			Chronic acidosis					
G. K.	Room air	3.96	2.50	1				
	3% CO_2	9.16	5.80	2.32	1.4	1.7	1.4	1.6
	5% CO_2	19.75	12.50	5.				
J. K.	Room air	5.22	3.13	1				
	3% CO_2	12.14	7.27	2.33	0.4	8.0	0 7	4.8
	5% CO_2	—	—	—				
W. E.	Room air	4.74	2.48	1				
	3% CO_2	10.98	5.75	2.31	2.0	1.4	1.0	2.0
	5% CO_2	—	—	—				
			Chronic alkalosis					
K. S.	Room air	3.63	2.20	1				
	3% CO_2	6.03	3.65	1.66	4.3	0.5	2.5	0.9
	5% CO_2	9.78	5.93	2.69				
J. T.	Room air	3.46	2.18	1				
	3% CO_2	5.51	3.46	1.59	6.6	0.3	3.2	0.7
	5% CO_2	8.70	5.47	2.51				

to double the effective alveolar ventilation in normal subjects is not much different from the value of 1.5 mm. Hg obtained by Haldane and Priestley (21) as early as 1905, using alveolar air samples. Gray (17) arrived at an average figure of 2.5 mm. Hg using 200 determinations reported in the literature under both steady and unsteady conditions, and based on alveolar air CO_2 tensions. The buffering capacity of normal human blood, measured *in vivo* under conditions of CO_2 inhalation and voluntary hyperventilation, has previously been reported by Shock and Hastings (26). It was found that when arterial hydrogen ion concentration is plotted against arterial CO_2 tension in mm. Hg, an almost linear relationship is obtained which is approximated in the hypercapnial range by the equation $(H^+)a = 0.652 \ PaCO_2 + 13.5$ (17). When plotted in the same way, the data obtained in this study using normal subjects also yield a linear relationship, the equation for which is $(H^+)a = 0.714 \ PaCO_2 + 8.3$.

The increase in physiological dead space to CO_2 (Bohr) accompanying the hyperpnea of CO_2 inhalation in normal subjects has long been recognized (24), and the magnitude of change observed in this study is comparable to that previously reported. Changes of this order of magnitude in the respiratory dead space with comparable increases in tidal volume have not been found using the nitrogen analysis technique (27). However, the size of the Bohr dead space to CO_2 is subject to changes in ventilation perfusion relationships in the lung, and may be increased, for example, when the lung contains appreciable numbers of well ventilated but poorly perfused alveoli. The increase in physiological dead space observed in this study with CO_2 inhalation is interpreted as suggesting a change in ventilation perfusion relationships under these conditions, perhaps secondary to changes in alveolar volume, or in respiratory air flow velocity, or possibly resulting from an effect of increased CO_2 tension on the pulmonary capillaries or bronchioles.

In evaluating the reduction in sensitivity to the carbon dioxide-hydrogen ion stimulus found in the Group III and IV emphysema patients, the possible effect of the hypoxic ventilatory drive at rest must be taken into consideration. As pointed out by Tenney (5), it might be expected that the diminution in anoxemia coincident with CO_2 inhalation would reduce this drive, and therefore the observed slope of the stimulus response curve might be less. As noted above, in these patients there was little or no further rise in arterial oxygen saturation with 5 per cent CO_2 inhalation as compared with 3 per cent CO_2, so that there was presumably no change in the degree of hypoxic drive between these two points on the stimulus response curve. Yet the slope of the curves between these two points did not differ from that between those determined at rest and on 3 per cent CO_2 inhalation, there being a consistent linear relationship of the three. Therefore changing hypoxic drive, such as may have occurred, did not modify the results obtained in any detectable way.

The reduction in sensitivity to the carbon dioxide-hydrogen ion stimulus found in patients with cor pulmonale secondary to emphysema might be ascribed to one or more of the following mechanisms: 1) Increased buffering capacity of the blood associated with an elevated plasma bicarbonate level or polycythemia; 2) failure of the chest bellows to respond adequately to the normal nervous stimuli; 3) the presence of congestive heart failure, chronic anoxemia, chronic acidosis, or chronic hypercapnia *per se*.

In 1920, Scott (3) suggested that the reduced ventilatory response to CO_2 inhalation which he observed in two patients with chronic emphysema might be explained on the basis of an increased buffering capacity of the blood secondary to a high bicarbonate level. This would result in a smaller increment in free hydrogen ion concentration on addition of a given amount of CO_2, and therefore a lesser ventilatory response. However, our measurements show that with CO_2 inhalation the blood hydrogen ion concentration in these patients actually increases more than in the normal group despite the presence of elevated blood bicarbonate and polycythemia. Thus the sensitivity is reduced as referred to either the CO_2 or hydrogen ion stimulus. Furthermore the absence of a reduced sensitivity in the patients with marked polycythemia associated with cyanotic congenital heart disease would argue against a specific effect of polycythemia in this regard.

The presence of mechanical ventilatory impairment did not condition the diminished sensitivity since a normal response was found in the Group II emphysema patients who suffered from com-

parable ventilatory defects. Likewise the presence of congestive heart failure *per se* does not account for the diminished sensitivity in cor pulmonale, since a reduction was also found in the Group III emphysema patients without congestive difficulty, and conversely one of the Group II emphysema patients with congestive failure on another basis had a normal response.

Since the anoxemia and acidosis of the patients with cor pulmonale were presumably of long duration, it appeared that a pertinent question in the present study was whether or not either chronic anoxemia or chronic acidosis might in themselves result in a diminished sensitivity to the carbon dioxide-hydrogen ion stimulus. Although the available evidence would indicate an increase in sensitivity to CO_2 associated with acclimatization to the anoxemia of altitude (*22*, 28), these observations were made over a period of days or weeks, and it was considered desirable to investigate the possibility that anoxemia of considerably longer duration might result in a diminished sensitivity. Likewise, although the observations of Peabody (19) suggested that if anything, an increased sensitivity to CO_2 was associated with renal failure, it seemed that the possible effects of known long-term acidosis should be explored. Accordingly, the patients with cyanotic congenital heart disease and slowly progressing uremia were selected to fulfill the criterion of chronicity. The absence of diminished sensitivity in either of these two patient categories has been taken as evidence that neither chronic anoxemia nor chronic acidosis in themselves condition the reduced response in patients with cor pulmonale.

The hypothesis that chronic hypercapnia itself might eventually lead to a diminished sensitivity to the carbon dioxide-hydrogen ion stimulus, although likely on the basis of exclusion, was more difficult of direct test. The hypercapnia of metabolic alkalosis seemed to offer the best clinical opportunity to explore this possibility, but long standing severe alkalosis and CO_2 retention are infrequently seen. The two patients with Cushing's syndrome therefore were chosen because of well-documented evidence of chronic metabolic alkalosis and CO_2 retention, there being no reason to believe that other metabolic derangements in these patients might condition the ventilatory response. As was the case with the emphysematous subjects, the diminution in sensitivity with reference to hydrogen ion precludes the possibility that increased buffering capacity of the blood resulted in a reduced response to CO_2. The findings are therefore taken as evidence that chronic hypercapnia *per se* leads to a diminution in sensitivity to the carbon dioxide-hydrogen ion stimulus. This thesis is further supported by the findings of Schäfer (*29*), who observed a reduced response to CO_2 in two normal men after four to six days residence in an atmosphere of 3 per cent CO_2.

Since both respiratory acidosis and metabolic alkalosis are associated with elevations in blood and tissue bicarbonate ion concentration as well as free carbon dioxide tension, no conclusion can be drawn from these observations as to which of these two excess quantities may lead to a reduced sensitivity, or whether both together do.

The findings in this study lend no support to the idea (30, 31) that an increased ventilatory response to CO_2 necessarily accompanies the condition of metabolic acidosis by reason of the lowered buffering capacity of the blood resulting in a greater increase in hydrogen ion concentration with the addition of a given amount of CO_2. Firstly, no increase in response to CO_2 was observed in two of the three patients with metabolic acidosis, and secondly, the patient demonstrating an increased response was found to have increased sensitivity in terms of hydrogen ion as well as CO_2. This latter observation would be consistent with the notion that an increase in sensitivity may accompany *chronic* hypocapnia, since this patient was observed to have a lowered venous serum CO_2 content on occasion for several years. On the other hand, if CO_2 acts as a respiratory stimulus only by increasing the hydrogen ion concentration in blood and tissue, then it might be expected that chronic metabolic acidosis would result in some diminution in sensitivity to the carbon dioxide-hydrogen ion stimulus as is observed with respiratory acidosis. The absence of such diminution with metabolic acidosis therefore may be construed as evidence favoring the theory that CO_2 acts as a specific stimulus.

We have interpreted the data as consistent with the hypothesis of CO_2 adaptation in prolonged hyper- and hypocapnia, and with the multiple factor theory of the chemical regulation of respiration advanced by Gray (17).

Little information is available presently as regards the degree of reversibility possible once a chronic diminution in sensitivity has become established. It is noteworthy that a diminished sensitivity was found in patient J. T. with Cushing's syndrome some four months after the venous serum CO_2 content had returned to normal.

The sequence of events in the natural history of chronic pulmonary emphysema leading to CO_2 retention is at present poorly defined. A tentative hypothesis suggested by these studies is the following: At some point in the course of the disease the ventilatory capacity becomes insufficient to eliminate the additional CO_2 produced during exertion, and transient rises in arterial CO_2 tension occur. Eventually these repeated bouts of hypercapnia result in a diminished sensitivity to the carbon dioxide—hydrogen ion stimulus, thus permitting an elevated CO_2 tension at rest though ventilatory capacity may be such that a normal CO_2 tension at an increased resting ventilation is possible. Furthermore, this elevated CO_2 tension at rest favors increased retention of bicarbonate by the kidney (32, 33) and thus a vicious cycle of increasing CO_2 retention and diminishing respiratory sensitivity is initiated.

The question of whether increasing CO_2 retention is associated with further diminution in sensitivity to the carbon dioxide-hydrogen ion stimulus cannot be answered with certainty at this time. Presumably serial observations on a number of patients would be needed. Lacking these, we have attempted to correlate the degree of sensitivity change with the amount of CO_2 retention in those patients having a reduced response to the carbon dioxide-hydrogen ion stimulus. In Figures 3 and 4 sensitivity is expressed both as the change in effective alveolar ventilation per square meter body surface area associated with 1 mm. Hg increase in arterial CO_2 tension, and as the change associated with unit increase in arterial hydrogen ion concentration. Sensitivity is plotted against the level of arterial CO_2 tension at rest. It is apparent that the sensitivity tends to diminish as the resting arterial CO_2 tension increases. No correlation between sensitivity and arterial serum CO_2 content in these same patients is demonstrable.

It is difficult to define possible therapeutic implications of these observations at this time. Nevertheless, it would seem reasonable to suppose that circumstances making for further CO_2 retention in some patients, such as acute pulmonary infection, vigorous exertion, or long continued oxygen therapy, would further accelerate a vicious cycle of diminishing sensitivity to CO_2, with resultant reduction in effective alveolar ventilation at rest, increased anoxemia and acidosis.

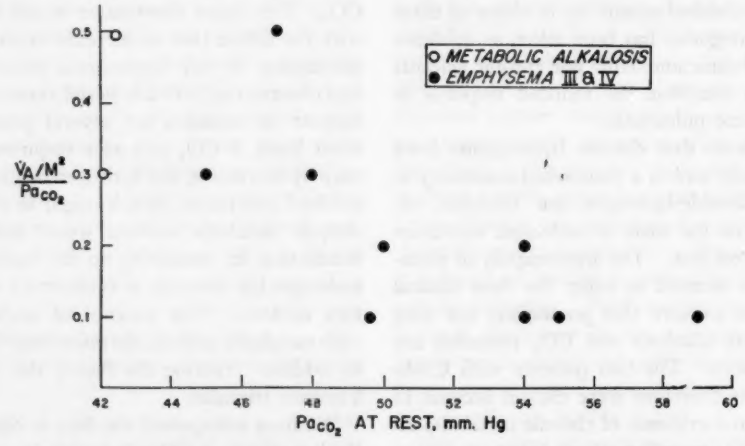

FIG. 3. SENSITIVITY TO THE CO_2 STIMULUS AS A FUNCTION OF THE DEGREE OF CARBON DIOXIDE RETENTION

Sensitivity appears on the ordinate in terms of increase in effective alveolar ventilation (liters BTPS per square meter body surface area) associated with 1 mm. Hg rise in arterial CO_2 tension. On the abscissa is plotted arterial CO_2 tension at rest.

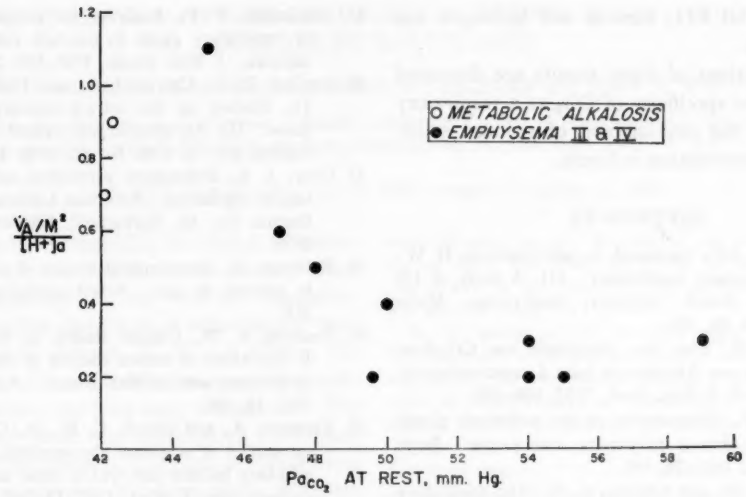

FIG. 4. SENSITIVITY TO THE HYDROGEN ION STIMULUS AS A FUNCTION OF THE DEGREE OF CARBON DIOXIDE RETENTION

Sensitivity appears on the ordinate in terms of increase in effective alveolar ventilation (liters BTPS per square meter body surface area) associated with unit rise in arterial hydrogen ion concentration (billionths of moles per liter). On the abscissa is plotted arterial CO_2 tension at rest.

SUMMARY AND CONCLUSIONS

1. In 12 normal subjects and 21 patients, sensitivity of the respiratory nervous regulatory mechanism to the carbon dioxide-hydrogen ion stimulus has been determined by relating effective alveolar ventilation to changes in arterial CO_2 tension and hydrogen ion concentration induced by CO_2 inhalation.

2. Six patients with chronic pulmonary emphysema and cor pulmonale were found to have a markedly reduced sensitivity to the carbon dioxide-hydrogen ion stimulus as compared with the normal.

3. Several mechanisms of possible importance in conditioning the reduced ventilatory response to CO_2 in the patients with cor pulmonale have been explored. They are: (a) increased buffering capacity of the blood associated with an elevated plasma bicarbonate level or polycythemia; (b) failure of the chest bellows to respond adequately to the normal nervous stimuli; (c) the presence of congestive heart failure, chronic anoxemia, chronic acidosis, or chronic hypercapnia *per se*.

4. The diminished response to CO_2 in the patients with cor pulmonale could not be accounted for on the basis of increased blood buffering capacity since the sensitivity was found to be reduced relative to the hydrogen ion as well as to the CO_2 stimulus.

5. Four patients without CO_2 retention but having chronic pulmonary emphysema and a mechanical ventilatory defect similar to that of the cor pulmonale group were found to have normal sensitivity to the carbon dioxide-hydrogen ion stimulus.

6. The diminished sensitivity in patients with cor pulmonale was not dependent upon the presence of congestive heart failure since three patients without congestive difficulty but having emphysema and CO_2 retention were found to have a reduced sensitivity.

7. No reduction in sensitivity was found in three patients with chronic anoxemia secondary to cyanotic congenital heart disease, nor in three patients with chronic metabolic acidosis associated with renal failure.

8. In two patients with chronic metabolic alkalosis and CO_2 retention, a diminished sensitivity was demonstrated.

9. It is concluded that chronic hypercapnia *per se* results in a diminished sensitivity to the CO_2 inhalation stimulus, which is associated with a rise

in both arterial CO_2 tension and hydrogen ion concentration.

10. Implications of these results are discussed in terms of the specificity of CO_2 as a respiratory stimulus and the development of reduced sensitivity in emphysematous subjects.

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MODIFICATION OF THE RESPIRATORY RESPONSE TO CARBON DIOXIDE BY SALICYLATE

By JAMES K. ALEXANDER,[1, 2] HAROLD F. SPALTER,[3] AND JOHN R. WEST †

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Cardio-pulmonary Laboratory, Presbyterian Hospital, New York, N. Y.)

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It is well known that CO_2 inhalation is associated with increased ventilation in normal man, and the sensitivity of the nervous regulatory mechanism to this physiological stimulus has been reasonably well defined (1–3). Likewise the clinical entity of hyperventilation associated with salicylate toxicity has long been recognized. The study reported here was designed to investigate one aspect of the effect of salicylate on respiration. Specifically an attempt has been made to determine whether salicylate might induce changes in the sensitivity of the nervous regulatory mechanism to the stimulus of CO_2 inhalation.

The approach has been essentially the same as that reported previously (3), namely, the correlation of arterial blood CO_2 tension and hydrogen ion concentration with effective alveolar ventilation when changes are induced by increased amounts of CO_2 in the inspired air.

MATERIAL AND METHODS

Three normal medical students were studied in the fasting state. Two studies each were made on two subjects and three on the third, each study extending over the period of one morning. Three sets of observations were made both before and after salicylate ingestion in order to determine three points on each of the stimulus response curves to be constructed. The observations were made while the subjects were breathing room air, 3 per cent CO_2 in air, and 5 per cent CO_2 in air in that order. The experimental procedure and apparatus used were the same as those previously employed in a larger group of normal subjects (3).

Single doses of 1.8 to 2.4 grams of acetyl salicylic acid were given orally in the form of "Bufferin®" [4] tablets approximately one and one-half to two hours before the second study was done, and blood for salicylate levels was drawn at the time the observations on ventilation and arterial blood were made.

In the normal subject a steady state as determined by consistency of respiratory quotient values, pulmonary ventilation, and respiratory frequency is obtained only after 25 to 30 minutes of uninterrupted breathing of a carbon dioxide gas mixture in the concentrations used (3, 4). Therefore, expired gas was collected during the last three minutes of 26 to 33-minute periods of continuous inhalation of the two CO_2 mixtures. During the middle minute of this three-minute period, arterial blood samples were steadily drawn from an indwelling needle in a brachial artery.

The analytical methods used have been described previously (3). Since the values for arterial CO_2 tension were partially derived from the blood pH, and the changes observed were relatively small, special attention was given to the determination of arterial blood pH in order to insure the greatest possible accuracy permitted by the technique employed. Duplicate determinations were carried out in every instance within five minutes after the blood had been removed from the vessel. Readings were made at 38° C. using a Cambridge glass electrode pH meter, transfer from vessel to electrode being accomplished anaerobically. Two standard phosphate buffer solutions of pH 7.18 and 7.58 at 38° C. (5) were used for reference, and duplicate blood determinations were required to check within ± .005 pH units. In this way changes in pH were measured accurately within .01 pH unit. Blood salicylate levels were determined colorimetrically (6). The usual 'alveolar' equation was utilized for the calculation of mean alveolar oxygen tension (7) when room air was inhaled, and in modified form (3) when CO_2 was added to the inspired air. Calculated values for whole blood buffer base were obtained from the Singer-Hastings nomogram (8).

If it can be assumed that in a steady state a consistent relationship or equilibrium exists between the concentration of certain chemical agents in the arterial blood and their intra-and/or extracellular concentrations acting at receptor or integrative levels on the nervous mechanism regulating respiration, then simultaneous measurement of arterial concentrations and pulmonary ventilation under varying conditions will permit the estimation of a stimulus response curve.

In analyzing the results of the study, the assumed physiological stimuli to respiration, *i.e.*, CO_2 tension and

[1] Present address: Department of Medicine, Baylor University College of Medicine, Houston, Texas.

[2] Supported in part by a gift from the Charles A. Frueauff Foundation.

[3] Fourth year student, Columbia University College of Physicians and Surgeons.

† Dr. West died June 29, 1954.

[4] Acetyl salicylic acid with aluminum glycinate and magnesium carbonate, Bristol Myers Co.

hydrogen ion concentration in the arterial blood, have been plotted against effective alveolar ventilation. Effective alveolar ventilation has been taken as the total pulmonary ventilation minus the physiological dead space ventilation (Bohr) to CO_2, assuming the arterial CO_2 tension to be equivalent to the mean alveolar tension. Alveolar ventilation has been utilized in constructing the stimulus response curves rather than the total pulmonary ventilation since it is the former which determines the tension of CO_2 in the arterial blood perfusing the receptor sites of the neural regulatory mechanism.

RESULTS

As indicated by the consistency of the respiratory exchange ratios,[5] a reasonably steady state was achieved in all three subjects during CO_2 inhalation in the control periods and following salicylate ingestion (Table I). In addition, relatively constant levels of salicylate ranging from 12 to 16 mg. per cent were obtained in the blood throughout the entire period of study in each subject (Table I). The latter findings were essentially the same as those of Smith, Gleason, Stoll, and Ogorzalek (6), who observed that after single 2 gram oral doses of acetyl salicylate the maximum plasma salicylate level was reached about one and one-half to two hours after ingestion and remained relatively constant for six hours thereafter.

The control observations (Tables I, II) on the ventilatory response to the increases in arterial CO_2 tension and hydrogen ion concentration associated with CO_2 inhalation were comparable to those made on a larger number of normal subjects. For a detailed discussion of the changes in pulmonary ventilation and arterial blood in normal subjects under these circumstances, the reader is referred to a previous report (3).

[5] Respiratory exchange ratio of expired air is used here as synonymous with respiratory quotient.

TABLE I

Physiologic data relative to pulmonary ventilation at rest and following salicylate ingestion *

Subject	State	Age	Sex	B.S.A.†	Dose, ASA‡	Blood salicylate level	Inspired gas mixture	Minutes on mixture	f	R_E	V_T	V_D§	V_E	V_A	PA_{O_2}
					grams	*mg. %*									
B. C.	Rest	23	M	2.06			Rm. air	—	13	0.75	528	200	6.87	4.27	95
							3% CO_2	27	15.3	0.85	738	257	11.32	7.38	121
							5% CO_2	30	26	0.85	1,075	564	27.95	13.28	130
	Post ASA				2.4	14.9	Rm. air	—	13	0.83	545	166	7.08	4.92	101
						16.4	3% CO_2	28	17	0.86	830	238	14.11	10.03	125
						16.4	5% CO_2	28	26	0.73	1,238	374	32.23	22.50	134
H. S.	Rest	24	M	1.90			Rm. air	—	8.3	0.86	788	269	6.57	4.33	102
							3% CO_2	26	12.3	0.83	951	300	11.72	8.02	118
							5% CO_2	26	23	0.75	1,433	745	33.0	15.84	129
	Post ASA				1.8	14.1	Rm. air	—	15	0.83	561	249	8.42	4.68	102
						13.0	3% CO_2	30	20.5	0.77	665	196	13.63	9.60	124
						13.3	5% CO_2	27	28	0.92	1,377	393	38.56	27.50	137
G. S.	Rest	23	M	1.92			Rm. air	—	9	0.75	646	211	5.81	3.92	93
							3% CO_2	33	11.3	0.75	1,024	338	11.61	7.78	117
							5% CO_2	26	13	0.65	1,843	935	23.96	11.81	125
	Post ASA				1.8	11.8	Rm. air	—	9.3	0.81	706	245	6.59	4.30	97
						12.8	3% CO_2	29	12	0.84	1,098	376	13.17	8.67	122
						11.8	5% CO_2	30	15.5	0.79	2,204	914	34.17	20.02	128
	Rest						Rm. air	—	7.7	0.77	693	211	5.33	3.71	95
							3% CO_2	28	9.3	0.70	1,000	218	9.30	7.27	114
							5% CO_2	28	11.3	0.79	2,070	839	23.42	13.94	127

* Symbols used are: f, respiratory frequency; R_E, respiratory exchange ratio, expired air; V_T, tidal volume, ml.; V_D, physiological (Bohr) dead space to CO_2 in ml., including both instrumental (60 ml.) and personal dead space; V_E, total pulmonary ventilation, liters per minute at body temperature and pressure saturated with water vapor; $\dot{V}_A$, effective alveolar ventilation, liters per minute BTPS; PA_{O_2}, calculated mean alveolar oxygen tension, mm. Hg.

† Body surface area, square meters.

‡ Acetyl salicylic acid.

§ For a discussion of changes observed with CO_2 breathing in normal subjects, see a previous report (3).

TABLE II

Physiologic data relative to the arterial blood at rest and following salicylate ingestion *

Subject	State	Inspired gas mixture	pHs	$(H^+)a$	Cs_{CO_2}	Pa_{CO_2}‡	Sa_{O_2}	Vc	$(B_B^+)b$
B. C.	Rest	Rm. air	7.40	39.8	58.7	41.5	97	41	47.5
		3% CO_2	7.39	40.7	61.5	44.5	99	41	49
		5% CO_2	7.36	43.6	62.7	48.5	100	41	49
	Post ASA†	Rm. air	7.41	38.9	58.3	40	95	40	48
		3% CO_2	7.41	38.9	59	40.5	98	41	48
		5% CO_2	7.39	40.7	61.5	44	100	42	48.5
H. S.	Rest	Rm. air	7.40	39.8	58.7	41.5	95	45	48
		3% CO_2	7.39	40.7	60	43.5	95	46	49
		5% CO_2	7.36	43.6	62.4	48	97	46	48.5
	Post ASA	Rm. air	7.42	38.0	59.9	40	97	47	50
		3% CO_2	7.42	38.0	60.6	41	99	47	50.5
		5% CO_2	7.40	39.8	62	44	100	47	50
G. S.	Rest	Rm. air	7.40	39.8	63.1	44.5	98	47	50
		3% CO_2	7.38	41.7	61.8	46	97	46	50
		5% CO_2	7.34	45.7	63	51	100	47	49
	Post ASA	Rm. air	7.40	39.8	61.8	44	100	45	50
		3% CO_2	7.38	41.7	61.1	45	99	47	49
		5% CO_2	7.35	44.6	60.8	48	98	48	49
	Rest	Rm. air	7.40	39.8	60.4	42.5	96	48	49
		3% CO_2	7.39	40.7	61.2	44.5	97	48	50
		5% CO_2	7.36	43.7	61.5	48	99	49	50

* Symbols used are: pHs, serum pH; $(H^+)a$, hydrogen ion concentration, billionths of moles per liter; Cs_{CO_2}, serum CO_2 content, vol. per cent; Pa_{CO_2}, CO_2 tension, mm. Hg; Sa_{O_2}, oxygen saturation; Vc, hematocrit; $(B_B^+)b$, calculated whole blood buffer base, mEq./L.

† Acetyl salicylic acid.

‡ In calculating Pa_{CO_2} from the Henderson-Hasselbalch relationship, pK′ for carbonic acid in blood serum was taken as 6:10, and the solubility coefficient of CO_2 in serum as 0.510 at 38° centigrade.

After salicylate ingestion there was little change in the values obtained at rest relative either to pulmonary ventilation or to arterial blood. Particular attention is called to the alveolar ventilation and arterial CO_2 tension in this regard (Tables I, II). The constancy of arterial blood buffer base values both before and after salicylate is taken as evidence that little renal compensation occurred during the period of respiratory acidosis induced by CO_2. With CO_2 inhalation, the relation between effective alveolar ventilation and arterial CO_2 tension or hydrogen ion concentration was essentially linear both before and after salicylate ingestion. As indicated by the slopes of the stimulus response curves, an increased sensitivity to the chemical stimulus in terms of either arterial CO_2 tension or hydrogen ion concentration was found in all subjects following salicylate

TABLE III

Sensitivity to the carbon dioxide-hydrogen ion stimulus

	Subject	Control	After salicylate	Percentage increase in sensitivity
Increase in effective alveolar ventilation, liters per minute BTPS, associated with a rise of 1 mm. Hg in arterial CO_2 tension.	B. C.	1.37	4.0	192
	H. S.	1.75	5.75	228
	G. S.*	1.44	3.88	170
Increase in effective alveolar ventilation, liters per min. BTPS, associated with a rise in arterial hydrogen ion concentration of 1 billionth of a mole per liter.	B. C.	2.2	8.2	272
	H. S.	2.8	11.4	307
	G. S.*	1.9	3.5	84

* Control values represent average of two studies.

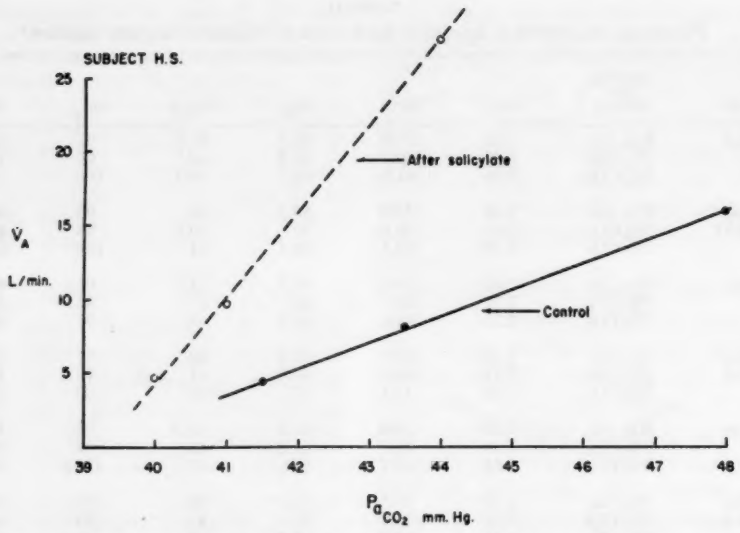

FIG. 1. RESPONSE TO THE CO_2 STIMULUS BEFORE AND AFTER SALICYLATE INGESTION

Effective alveolar ventilation in liters per minute (BTPS) on the ordinate is plotted against arterial CO_2 tension in mm. Hg on the abscissa to obtain the slopes of the stimulus-response or sensitivity curves.

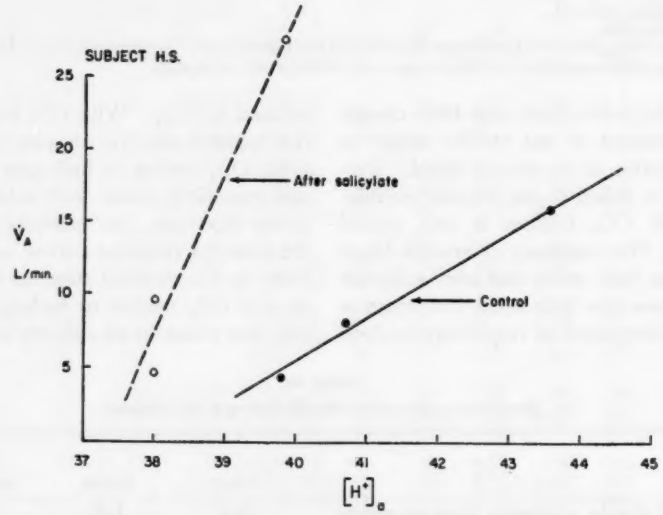

FIG. 2. RESPONSE TO THE HYDROGEN ION STIMULUS BEFORE AND AFTER SALICYLATE INGESTION

With arterial hydrogen ion concentration in billionths of moles per liter (see Table II) on the abscissa, sensitivity curves are constructed as indicated in Figure 1.

ingestion. The stimulus response curves of subject H. S. are shown in Figures 1 and 2. In Table III are shown the increments in effective alveolar ventilation associated with unit rise in arterial CO_2 tension and hydrogen ion concentration during the control observations and after salicylate, together with the percentage increase in sensitivity induced by salicylate in each subject. Even at the relatively low blood salicylate levels obtained, increases in sensitivity ranging from 84 per cent to 307 per cent occurred.

DISCUSSION

Possible mechanisms which have been suggested (9) to account for salicyl hyperpnea are (a) a direct stimulating effect upon the respiratory center, (b) a metabolic acidosis resulting from fixed acid degradation products of these compounds. In regard to the latter, recently reported studies by Singer (10) indicate that there may be accumulation of an as yet unidentified fixed acid in the blood with metabolic acidosis in the advanced phase of salicylate poisoning. However, it seems well established that the hyperpnea accompanying large therapeutic dosage or mild to moderate toxicity is associated with an uncomplicated respiratory alkalosis (9, 10).

The results of this study are interpreted as providing direct experimental evidence that an increase in the sensitivity of the nervous regulatory mechanism to normal chemical stimuli is at least one means whereby hyperventilation is induced by salicylates.

SUMMARY AND CONCLUSIONS

1. The sensitivity of the respiratory nervous regulatory mechanism to the CO_2 inhalation stimulus was determined in three normal subjects before and after salicylate ingestion, relating effective alveolar ventilation to arterial CO_2 tension and hydrogen ion concentration.

2. With blood salicylate levels of the order 12 to 16 mg. per cent, respiratory sensitivity to the carbon dioxide-hydrogen ion stimulus increased 84 to 307 per cent.

3. It is suggested that this increased sensitivity to a normal chemical stimulus is one mechanism whereby salicylates induce hyperventilation.

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THE ACIDIFYING EFFECT OF RUBIDIUM IN NORMAL AND POTASSIUM-DEFICIENT ALKALOTIC RATS[1]

By ARNOLD S. RELMAN, ARLENE M. ROY, AND WILLIAM B. SCHWARTZ

(From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine; and from the New England Center Hospital, and the Department of Medicine, Tufts College Medical School, Boston, Mass.)

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Rubidium, element number 37, is an alkali-metal found in small quantities in the earth's crust, and in trace amounts in human tissues (1). Listed immediately below potassium in the Group I elements of the periodic table, rubidium possesses many of potassium's physical, chemical, and physiological properties. These two elements have qualitatively similar effects on the contraction of the isolated heart (2), on the depolarization of nerve or muscle preparations (3, 4), and on the activity of certain intracellular enzyme systems (5, 6). The distribution of rubidium in human tissues resembles that of potassium (1, 7), and rubidium has been shown to be concentrated in certain plant cells (8) and in animal tissues (9, 10) with great rapidity. It has also been reported that addition of rubidium to a potassium-free diet will prevent the development in rats of most of the histological changes associated with potassium depletion (11).

The central purpose of the present work was to compare the effects of rubidium and potassium ions on acid-base balance in normal and in potassium-depleted rats. When given an alkaline-ash, potassium-deficient diet, rats develop an alkalosis (12), which can be rapidly corrected by administration of potassium chloride (13). The present data indicate that rubidium has an acidifying effect on extracellular fluid at least equal to, and under some circumstances clearly greater than, that of potassium.

METHODS AND MATERIALS

Male albino rats of the Sprague-Dawley strain were used in all experiments. Four types of experiments were carried out. In the first, the chloride salts of sodium, potassium, and rubidium were administered intraperitoneally in equivalent amounts for a period of six days to normal growing rats on a Purina Lab Chow diet. In the second experiment, weanlings were made alkalotic and potassium-deficient by the administration of an alkaline-ash, potassium-free diet, and then treated over a six-day period with equivalent amounts of sodium chloride, rubidium chloride, and potassium chloride. In a third experiment, a large quantity of rubidium chloride or potassium chloride was given in a period of one day to potassium-deficient alkalotic rats. In the final experiment, potassium-deficient alkalotic rats were nephrectomized and then injected with sodium chloride, rubidium chloride or potassium chloride.

Animals were kept in separate cages. Food and water were allowed *ad libitum*. All injections were made intraperitoneally. Blood specimens were taken directly from the abdominal aorta in oiled heparinized syringes after the animal had been lightly anesthetized with Sodium Amytal® (20 mg. per 100 gm.). The pH of whole blood was determined anaerobically at room temperature in a research model Cambridge pH meter and corrected to 37° C. (.01 pH unit per degree). It should be noted that blood pH in rats is subject to relatively large fluctuations due to acute changes in respiratory activity immediately prior to and during exsanguination. For this reason, pH data are less reliable indices of acid-base disturbances in the rat than are determinations of total CO_2 content (13). Total CO_2 content of plasma was measured by standard manometric technique. The chloride content of plasma was determined by Volhard titration. Sodium and potassium were determined with a flame photometer. No analyses for rubidium or potassium could be done in the rats given rubidium since these ions could not be quantitatively determined in the presence of each other by any of the commercial flame photometers available to us, or by any of the standard titrimetric or gravimetric procedures suitable for analysis of either ion separately.

The details of each experiment follow:

Experiment I (Table I): Normal 90 to 100 gm. rats were divided into four groups. All animals were allowed to eat Purina Lab Chow and to drink tap water *ad libitum*. Those in Group 1 were controls and received no injections. Animals in Group 2 were given daily intraperitoneal injections of sodium chloride solution, Group 3

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TABLE I *

Effect of chronic administration of NaCl, KCl, and RbCl to normal rats

Group	No. rats	Blood pH	Plasma CO mM/L.	Plasma Potassium mEq./L.
1. Control	11	7.49±0.05	26.2±1.92	4.28±0.09
2. NaCl	13	7.45±0.04	25.5±1.92	4.33±0.35
3. KCl	11	7.45±0.04	25.3±1.71	4.40±0.32
4. RbCl	13	7.50±0.04	22.5±1.38	—

* In this table, and in all subsequent tables, values for blood analyses are given as the mean ± the standard deviation.

received potassium chloride solution and Group 4 rubidium chloride solution. Each animal received 3 mEq. per kgm. of the respective salt as a 200 mEq. per L. solution, and the injections were continued for six consecutive days so that a total of 18 mEq. per kgm. was administered. Animals were sacrificed on the seventh day, approximately 24 hours after Groups 2, 3, and 4 had received their last injection.

Experiment II (Table II): Ninety to one hundred gm. rats were given a potassium-free diet (Groups 2–6) identical in composition with that used by Cotlove, Holliday, Schwartz, and Wallace (14) except that the inorganic salt content was altered by removing calcium carbonate, dibasic sodium phosphate, and sodium chloride from the salt mixture and substituting a mixture of monobasic and dibasic calcium phosphate in a molar ratio of 1:2. This produced no change in the final calcium and phosphorus content (approximately 0.6 per cent each). Sodium, chloride, and bicarbonate ions were added to the diet in the drinking water, which contained 100 mEq. per L. sodium chloride and 50 mEq. per L. sodium bicarbonate. This provided an acid-ash, potassium-free diet, with adequate quantities of sodium and chloride and all the alkali provided entirely in the drinking water.[2] Over the entire range of solid (acid-ash) and water (alkaline-ash) intakes observed in our rats, the net intake on this regime was always alkaline. A control group of animals (Group 1) was given the same diet and drinking water, but potassium bicarbonate, 87 mm. per kgm. of diet, was added to their food.

At the end of sixteen days, some of the potassium-deficient rats were sacrificed (Group 2) and the rest of them were divided into four groups. All these latter animals were continued on the potassium-free diet, but they were given only distilled water to drink, thus removing all dietary bicarbonate, sodium, and chloride, and thereby converting the net intake to acid-ash. One group (Group 3) was given no other therapy. Another group (Group 4) was injected intraperitoneally with sodium chloride solution, another with potassium chloride (Group 5), and the last with rubidium chloride (Group 6), all in a dosage of 5 mEq. per kgm. per day for six consecutive days, using a 200 mEq. per L. solution. All of these animals, as well as the control group (Group 1) taking a complete diet, were sacrificed on the 23rd day, approximately 24 hours after Groups 4, 5, and 6 had received their last injection.

Experiment III (Table III): A group of 80 to 90 gm. rats was made alkalotic and potassium-deficient by 18 days of feeding with the potassium-free, alkaline-ash diet used by Cotlove, Holliday, Schwartz, and Wallace (14), and some (Group 1) were sacrificed at the end of this time. The remaining animals were then divided into two groups, one of which received intraperitoneal injections of potassium chloride solution (Group 2) and the other, rubidium chloride solution (Group 3). A total of 18 mEq. per kgm. was given in four equal doses during the 19th day, at a concentration of 100 mEq. per L. All animals in Groups 2 and 3 were sacrificed on the 20th day, approximately 18 hours after the last injection.

Experiment IV (Table IV): One hundred and fifty gm. rats were fed the diet used in Experiment II, with sodium chloride and sodium bicarbonate added to their drinking water, as before. A control group (Group 1), to whose diet potassium bicarbonate, 87 mm. per kgm. had been added, and a group of potassium-deficient animals (Group 2) were killed on the 15th day. The remainder of the potassium-deficient rats were nephrectomized on the 15th day under light Sodium Amytal® anesthesia (20 mg. per 100 gm.), and then divided into four groups.

[2] These changes were made in order to facilitate use of this diet in other experiments in which the relative quantities of dietary alkali (bicarbonate) and chloride were varied.

TABLE II

Effect of chronic administration of NaCl, KCl, and RbCl to K-deficient alkalotic rats

Group	No. rats	Blood pH	Plasma CO_2 mM/L.	Plasma Potassium mEq./L.
1. K-added control	14	7.41±0.02	26.2±1.65	4.04±0.26
2. K-deficient control	15	7.52±0.07	36.4±4.55	1.63±0.27
3. K-deficient followed by removal of alkali from drinking water	9	7.50±0.05	28.9±4.69	1.59±0.20
4. Same as Group 3, +NaCl	11	7.41±0.06	28.2±3.10	1.82±0.27
5. Same as Group 3, +KCl	15	7.41±0.05	26.9±2.65	3.58±0.65
6. Same as Group 3, +RbCl	13	7.38±0.08	19.5±4.23	—

TABLE II-A

P values for CO_2 data of Table II *

	Group 1 $(\bar{x})$ = 26.2 mM/L.	Group 2 $(\bar{x})$ = 36.4 mM/L.	Group 3 $(\bar{x})$ = 28.9 mM/L.	Group 4 $(\bar{x})$ = 28.2 mM/L.	Group 5 $(\bar{x})$ = 26.9 mM/L.	Group 6 $(\bar{x})$ = 19.5 mM/L.
Group 1. K-added control $(\bar{x})$ = 26.2 mM/L.		<.01	N.S.*	N.S.	N.S.	<.01
Group 2. K-deficient control $(\bar{x})$ = 36.4 mM/L.	<.01		<.01	<.01	<.01	<.01
Group 3. K-deficient followed by removal of alkali from drinking water $(\bar{x})$ = 28.9 mM/L.	N.S.	<.01		N.S.	N.S.	<.01
Group 4. Same as Group 3, +NaCl $(\bar{x})$ = 28.2 mM/L.	N.S.	<.01	N.S.		N.S.	<.01
Group 5. Same as Group 3, +KCl $(\bar{x})$ = 26.9 mM/L.	N.S.	<.01	N.S.	N.S.		<.01
Group 6. Same as Group 3, +RbCl $(\bar{x})$ = 19.5 mM/L.	<.01	<.01	<.01	<.01	<.01	

* P > .05.

One group (Group 3) was given no therapy and allowed no food or water. They were sacrificed four hours after nephrectomy. Three groups were injected with a total of 8 mEq. per kgm. of sodium (Group 4), potassium (Group 5), or rubidium (Group 6). These cations were given in two equal doses at concentrations of 126 mEq. per L. as the chloride salt, together with 24 mEq. per L. as the bicarbonate salt.[3] The first dose was given immediately after nephrectomy and the second dose two hours later. The animals were sacrificed two hours after the second dose, *i.e.*, four hours after nephrectomy. During this time they were allowed no food or water.

To determine the extent of uptake of the administered rubidium and potassium, the fluid remaining in the peritoneal cavity (usually 1 to 4 cc.) was analyzed for sodium (and also for potassium in the animals given this cation) in several rats of each group. In all instances the concentrations of these cations in the peritoneal fluid were essentially equal to those in plasma. These results indicate cellular uptake of almost all the administered potassium, and uptake of at least the largest part of the administered rubidium.

TABLE III

Effect of acute administration of KCl+RbCl to alkalotic, K-deficient rats

Group	No. rats	Blood pH	Plasma CO_2 mM/L.	Plasma Potassium mEq./L.
1. K-deficient control	11	7.48±0.05	34.1±3.94	1.68±0.09
2. KCl	7	7.40±0.04	29.8±3.05	4.17±1.10
3. RbCl	5	7.37±0.03	29.1±3.10	—

[3] Bicarbonate was added, as in the experiments of Orloff, Kennedy, and Berliner (15), to minimize changes in CO_2 content due solely to dilution by the volume of administered fluid.

RESULTS

Experiment I: Chronic administration of sodium chloride, potassium chloride, and rubidium chloride to normal rats

All the injected animals seemed to tolerate the various chloride salts without difficulty and all gained approximately the same amount of weight. The chemical data are summarized in Table I. It is seen that injections of sodium chloride and potassium chloride had no significant effect on blood pH or plasma content of carbon dioxide or potassium. The group treated with rubidium chloride showed a small reduction in plasma CO_2 content which was statistically significant [4] when referred to any of the other groups, including the

[4] In the reporting of results, and in the subsequent discussion of the data, whenever a difference between two group means is said to be "significant," it will be understood that the t-test yields a value of "p" less than .01.

TABLE IV

Effect of nephrectomy alone, and of nephrectomy+NaCl, KCl, and RbCl in alkalotic, K-deficient rats

Group	No. rats	Blood pH	Plasma CO_2 mM/L.	Plasma Na mEq./L.	Plasma K mEq./L.	Plasma Cl mEq./L.
1. Normal diet	14	7.54±.06	26.7±1.3	141.0±1.6	3.52±.38	103.5±2.8
2. K-deficient	22	7.58±.08	37.6±3.8	142.2±2.3	1.75±.34	90.2±2.3
3. K-deficient+nephrectomy	11	7.55±.05	31.5±2.1	142.8±1.9	1.84±.46	89.6±2.4
4. K-deficient+nephrectomy+NaCl	20	7.60±.07	29.5±3.1	143.7±2.9	1.79±.23	96.7±4.6
5. K-deficient+nephrectomy+KCl	16	7.55±.06	27.1±2.4	141.8±2.5	2.90±.79	100.0±6.3
6. K-deficient+nephrectomy+RbCl	17	7.54±.05	25.7±4.3	141.9±3.8	—	104.5±5.9

TABLE IV-A

P values for CO_2 data of Table IV

	Group 1 ($\bar{x}$) =26.7 mM/L.	Group 2 ($\bar{x}$) =37.6 mM/L.	Group 3 ($\bar{x}$) =31.5 mM/L.	Group 4 ($\bar{x}$) =29.5 mM/L.	Group 5 ($\bar{x}$) =27.1 mM/L.	Group 6 ($\bar{x}$) =25.7 mM/L.
Group 1. Normal diet ($\bar{x}$) =26.7 *mM/L.*		<.01	<.01	<.01	N.S.	N.S.
Group 2. K-deficient ($\bar{x}$) =37.6 *mM/L.*	<.01		<.01	<.01	<.01	<.01
Group 3. K-deficient+nephrectomy ($\bar{x}$) =31.5 *mM/L.*	<.01	<.01		N.S.	<.01	<.01
Group 4. K-deficient+nephrectomy+NaCl ($\bar{x}$) =29.5 *mM/L.*	<.01	<.01	N.S.		>.02 <.03	<.01
Group 5. K-deficient+nephrectomy+KCl ($\bar{x}$) =27.1 *mM/L.*	N.S.	<.01	<.01	>.02 <.03		N.S.
Group 6. K-deficient+nephrectomy+RbCl ($\bar{x}$) =25.7 *mM/L.*	N.S.	<.01	<.01	<.01	N.S.	

control. The mean pH of none of the treated groups was significantly different from the control, but as pointed out earlier, little weight should be attached to these findings.

Experiment II: Chronic administration of sodium chloride, potassium chloride, and rubidium chloride to potassium-deficient alkalotic rats

The chemical data are summarized in Table II. Sixteen days of a potassium-free diet, with alkali in the drinking water, resulted in a severe metabolic alkalosis with hypokalemia (Group 2). Mean CO_2 content in this group was 36.4 mM per L., approximately 10 mM per L. higher than the controls. Removal of alkali from the drinking water and continuation of the now acid-ash, potassium-free intake for another six days (Group 3) produced a significant reduction in CO_2 content to 28.9 mM per L., but the mean potassium concentration was not significantly different from the sixteen-day alkalotic group.

Treatment for six days with a total of 30 mEq. per kgm. of sodium chloride, combined with removal of sodium chloride and sodium bicarbonate from the drinking water (Group 4), had no more effect on mean CO_2 content than the single maneuver of substituting distilled water for the alkaline drinking water. Removal of alkali from the water, combined with treatment with potassium chloride (Group 5), resulted in a mean CO_2 content of 26.9 mM per L., which was not significantly different from the normal controls (Group 1) or from either Group 3 or Group 4. Plasma potassium was restored to almost normal levels.

The group of rats treated with rubidium chloride injections and removal of sodium chloride and sodium bicarbonate from their drinking water (Group 6) showed the largest reduction in mean CO_2 content (19.5 mM per L. ± 4.23). This value was significantly lower than that found in any of the other groups, including even the normal controls.

The "p" values for the differences among the mean CO_2 contents of the various groups are listed in Table II-A.

The data on mean pH value showed relatively smaller changes than were observed in total CO_2 content, but in general, the changes were in the same direction.

The general appearance of the animals in the potassium and rubidium groups was the same. These rats began to gain weight and behaved more normally than those in Groups 3 and 4, which continued to lose weight.

Experiment III: Acute administration of potassium chloride and rubidium chloride to alkalotic potassium-deficient rats

Table III presents the results of the experiment, in which 18 mEq. per kgm. of potassium chloride or rubidium chloride was administered to alkalotic potassium-deficient rats over a 24-hour period. This treatment produced an approximately equal fall in CO_2 content in both groups. The differences between the untreated group and each of the treatment groups was significant at the 3 per cent level. Blood pH was reduced significantly and almost equally in both groups.

Experiment IV: Acute administration of sodium chloride, potassium chloride, and rubidium chloride to nephrectomized potassium-deficient alkalotic rats

Table IV summarizes the data from the nephrectomy experiment. Fourteen days on a potassium-deficient, alkaline-ash diet (Group 2) produced the expected rise in CO_2 content (37.6 mM per L.), and fall in potassium (1.75 mEq. per L.) and chloride (90.2 mEq. per L.) concentration. Four hours after bilateral nephrectomy (Group 3) there was a significant drop in CO_2 content to 31.5 mM per L., but no significant change in chloride, sodium or potassium; bicarbonate had therefore been replaced by the anions of unmeasured endogenous acids.

Nephrectomy plus intraperitoneal administration of sodium chloride and bicarbonate (Group 4) effected a slight but not significant drop in CO_2 content to 29.5 mM per L., and a larger and significant rise in plasma chloride to 96.7 mEq. per L. Sodium and potassium concentrations were essentially unchanged.

Treatment of nephrectomized animals with equivalent amounts of potassium chloride and bicarbonate (Group 5) resulted in a further drop in CO_2 content to 27.1 mM per L., which was now significantly lower than the CO_2 content of the Group 3 animals and essentially equal to the CO_2 in the normal animals. Chloride was slightly, though not significantly, higher than in the sodium-treated group, but sodium concentration was unchanged. As expected, plasma potassium was significantly higher than in any of the other potassium-deficient groups, but it was still below the normal level.

Administration of rubidium chloride and bicarbonate to nephrectomized rats (Group 6) produced the largest fall in CO_2 content (25.7 mM per L.), and the greatest rise in chloride concentration (104.5 mEq. per L.). These mean values, which were essentially equivalent to the normal (Group 1), differed significantly from the comparable data in both the untreated nephrectomized rats (Group 3) and the rats treated with sodium chloride (Group 4), but they were not significantly different from the means in the potassium group.

Table IV-A summarizes the "p" values for the differences among mean CO_2 contents in this experiment.

For the most part changes in pH tended to follow those in carbon dioxide, but they were usually small and not significant. Occasionally, pH and CO_2 appeared to deviate (Table I, group 4, Table IV, group 4).

DISCUSSION

In acute studies rubidium appears to be as effective as potassium in its ability to correct the alkalosis of potassium-deficiency, but the results of more chronic administration of rubidium indicate that this cation is more than merely a physiological substitute for potassium in its influence on acid-base metabolism. Prolonged administration

of rubidium chloride to normal or potassium-deficient alkalotic animals produces a distinct extracellular acidosis, with bicarbonate values significantly below those of normal controls or those of animals given equivalent amounts of sodium or potassium chloride.

In potassium-deficient alkalotic animals, cellular accumulation of administered potassium is associated with displacement of intracellular hydrogen ions and subsequent reduction in extracellular bicarbonate concentration (13, 15). Since there is evidence that animal tissues can also accumulate large quantities of administered rubidium ions (9, 16), it is reasonable to assume that the acidifying effects of rubidium in the present experiments were accomplished, at least in part, by a similar displacement of cellular hydrogen.

In the nephrectomy experiment exchange of rubidium for hydrogen was probably the major factor in the fall in bicarbonate concentration. Other possible explanations seem unlikely. Cellular uptake of administered cation with bicarbonate (15) could not account for more than a small moiety of the rubidium estimated to have penetrated cells. Cellular extrusion of molecular acids seems unlikely because the plasma concentration of unmeasured anions (estimated by the difference between sodium and the sum of CO_2 and chloride) was reduced rather than increased. Dilution of extracellular fluid by the volume of administered rubidium chloride solution would account for a fall of only 1 to 2 mM per L. in plasma CO_2 content, which was the change observed in the group treated with sodium chloride.

In the chronic experiments, extrusion of intracellular hydrogen was probably also the primary mechanism for reduction of extracellular bicarbonate, but the fall in bicarbonate to sub-normal levels in the rubidium animals requires further explanation. Compared to potassium, rubidium may be more slowly excreted from the body and hence penetrate cells to a greater extent; it may be preferentially taken up by muscle cells, or in some manner it may displace more intracellular hydrogen and less intracellular sodium than does an equivalent amount of potassium. On the other hand, the acidifying action of rubidium in the chronic experiments, particularly with the normal animals, may partly be ascribable to an effect of the cation on renal tubular transport of bicarbonate. There is evidence (17) that an excess of potassium ions reduces bicarbonate reabsorption and tends thereby to lower plasma bicarbonate. Conceivably rubidium might act in a similar, though more effective, manner. Evaluation of such speculations would require urine or tissue analyses or balance studies, all of which were beyond the scope of the present studies.

The present experiments not only suggest that replacement of intracellular cation by rubidium is as effective as replacement by potassium in the abolition of extracellular alkalosis, but they also demonstrate that the latter state may be ameliorated merely by the addition of acid, without restoration of normal cellular cation composition. It is a common clinical aphorism that alkalosis associated with potassium deficiency is "resistant" to any therapy other than replacement of the intracellular cation. However, in Experiment II (Group 3, Table II), it was possible to lower plasma bicarbonate to a value not significantly higher than the normal controls (Group 1) or the potassium-treated group (Group 5) simply by removing alkali from the drinking water and allowing the animals to continue ingestion of an acid-ash diet for six days. These animals received no potassium at any time during the experiment, and their final mean plasma potassium concentration was identical with that of the untreated potassium-deficient alkalotic group (Group 2).

The effects of acid also explain why, in Experiments II and IV, potassium does not appear to be much more effective than sodium in the correction of potassium-deficiency alkalosis. In these experiments an acid-ash intake (Experiment II) or the accumulation of endogenous acids after nephrectomy (Experiment IV) had reduced blood CO_2 content in the sodium treated animals so nearly to normal that treatment with potassium could not be expected to have more than a slight additional effect.

SUMMARY AND CONCLUSIONS

1. Acute administration of rubidium chloride to potassium-deficient alkalotic rats is as effective in reducing extracellular bicarbonate concentration as the administration of equivalent amounts

of potassium chloride. The data suggest that rubidium, like potassium, exerts its acidifying effect by exchanging for intracellular hydrogen.

2. Administration of rubidium chloride for six days to normal or potassium-deficient alkalotic rats produces a metabolic acidosis, while equivalent amounts of potassium do not lower bicarbonate concentrations below normal levels. Possible mechanisms by which rubidium produces metabolic acidosis are discussed.

3. Alkalosis associated with potassium depletion can be corrected without administration of intracellular cation simply by increasing the ratio of acid to alkali in the diet.

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ANNOUNCEMENTS OF MEETINGS

The 47th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, N. J., on Monday, May 2, 1955, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 a.m. at the Steel Pier Theater.

The annual meeting of the Association of American Physicians will be held at the Steel Pier Theater on Tuesday, May 3, and at the Chalfonte-Haddon Hall on Wednesday morning, May 4, 1955.

A STUDY OF THE RELATIONSHIP BETWEEN RADIOACTIVE SODIUM CLEARANCE AND DIRECTLY MEASURED BLOOD FLOW IN THE BICEPS MUSCLE OF THE DOG

By THEODORE C. PRENTICE, ROBERT R. STAHL, NORMAN A. DIAL, and FRANK V. PONTERIO

(From the Division of Surgery, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.)

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Since the introduction by Kety in 1949 (1) of the radioactive sodium clearance method for measuring regional blood flow in muscle, much work using this method has been published. The results have been varied, and conflicting in many respects. Some have supported the contention that the rate of sodium clearance is a good index of local muscle blood flow (2, 3), others have not (4, 5). Perhaps the dominant conclusions one draws from existing data are that: 1) There is a wide range of values for sodium clearance rate from muscle in the resting human; 2) there is a large overlap between clearance rates from a normal group and from a group with known peripheral vascular disease; and 3) nevertheless for any given individual those influences such as exercise, drugs, and temperature, which have been noted to increase or decrease muscle blood flow, usually cause parallel changes in the rate of sodium clearance from muscle.

The problem arises then as to whether the wide range of clearance values noted in normals, and the considerable overlap found between normal and pathologic material, reflect actual variation in regional flow rates or are due to inherent errors in the clearance method as an index of flow.

To further elucidate this problem and to test as rigorously as possible the accuracy and dependability of the sodium clearance method as a measurement of total blood flow, a series of direct and simultaneous comparisons between total blood flow through muscle and sodium clearance therefrom was carried out.

Any conclusions concerning blood flow as related to radioactive sodium clearance from this study must of necessity refer to *total* blood flow since that was the quantity measured. Effective blood flow, which refers to the fraction of total blood flow going through the capillaries, cannot be directly measured at the present time. Therefore any conclusions relating the clearance rate of radioactive sodium to effective blood flow (capillary circulation) are in the realm of speculation.

METHODS

Ten experiments were performed to determine the relationship between the clearance rate of radioactive sodium from an injected intramuscular site and the blood flow through the muscle. Dogs used in this experiment, ranging in weight from 12.7 to 23 Kg., were anesthetized with Sodium Nembutal.®

A large incision was made in the skin of the foreleg extending from the anterior superior surface of the shoulder joint, distally and somewhat medially, to the elbow joint. The skin and subcutaneous tissue were reflected and the underlying muscle was exposed. Several large muscles, serratus ventralis, brachiocephalicus, pectoralis superficialis, and pectoralis profundus, were divided and reflected so as to expose the biceps muscle. This also allowed for exposure of the brachial artery and vein which were about one centimeter medially and along the longitudinal axis of the muscle. The brachial artery and vein and their branches and tributaries were carefully dissected and freed of their connective tissue attachments. In most of the animals three pairs of nutrient vessels supplied the biceps muscle (Figure 1). The artery and vein which entered the middle portion of the muscle on its medial aspect were the largest in caliber and these vessels were the ones used for perfusion. The smaller pairs of vessels at the proximal and distal portions of the muscle were ligated. All other branches of the brachial artery and tributaries of the brachial vein were ligated and divided. A cannula (C_T) was placed in the brachial vein and this vein was ligated just distal to the point where the vein from the biceps muscle joined it. This made possible the collection of the venous outflow from the biceps muscle. The experimental dogs received heparin approximately

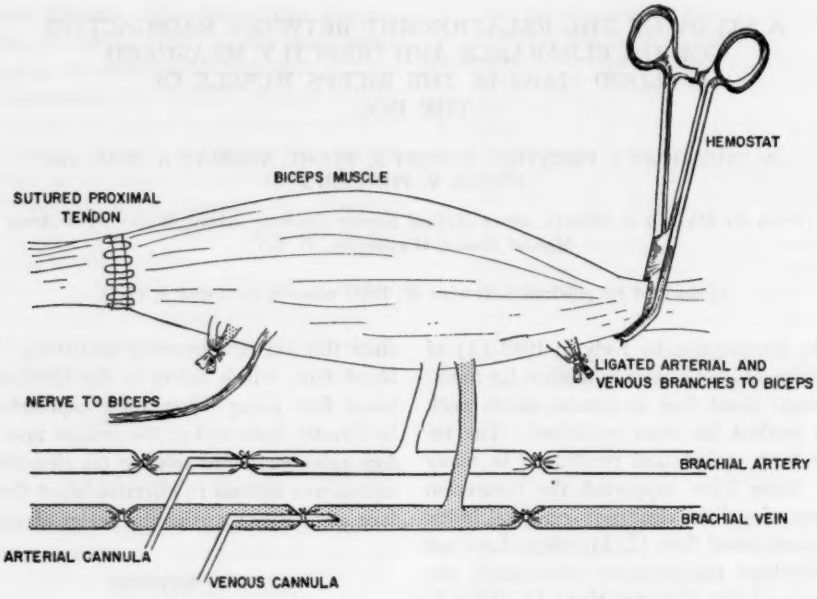


FIG. 1. SCHEMATIC DIAGRAM OF LOCAL ANATOMY AND DISSECTION

4.5 mg. per Kg. of body weight just prior to the insertion of the venous cannula. A cannula (C_A) was placed in the brachial artery which was ligated just distal to the arterial branch to the biceps. The arterial cannula was then connected to the perfusion pump with Tygon tubing.

In each experiment the venous outflow from the biceps muscle was measured immediately after cannulation of the brachial vein and just prior to the cannulation of the brachial artery. In all instances a relatively large rate of flow was found. These values, however, were not reported since they did not appear directly relevant to the experiment and ligation of numerous branches of the brachial artery would tend to divert more blood to the biceps muscle.

The nerve supply to the biceps was left intact except in Experiment No. 2 when it was inadvertently severed during dissection.

Both the tendon of origin and tendon of insertion of the biceps muscle were severed. This was necessary so as to obliterate any blood supply reaching the muscle by way of the tendons. At this point in the dissection the entire muscle could be lifted and completely separated from the extremity except for the arterial branch, venous tributary, and the nerve supplying the biceps. The proximal tendon, the tendon of origin, was sutured together again. The more distal tendon, the tendon of insertion, was short; the tendinous portion of the muscle was clamped with a small hemostat. The hemostat was anchored to the extremity with thread so as to maintain anatomic relations and relative muscle stretch.

Donor dogs were anesthetized, heparinized (approximately 4.5 mg. per Kg. of heparin), and bled into an open reservoir. In all but Experiment Nos. 1, 3, and 4 this blood was passed through an oxygenating device. The oxygenating device for the blood consisted of a Vigreaux distillation column to which a glass joint and side tube was attached near the bottom. Oxygen was allowed to enter through this side tube. Blood was poured into the column at the top and bubbles of oxygen passed up through the tube as the column of blood descended. This method of oxygenation resulted in very little hemolysis as demonstrated by the plasma hemoglobin values before and after passage through the oxygenating column. Prior to this passage of the blood through the column, 5 to 10 ml. of heparin was added. This blood was then placed in two open reservoirs A and B (Figure 2).

A Phipps and Bird Infusion Pump was utilized to establish a relatively constant blood flow through the muscle (Figure 2). This pump pushes a standard 50 ml. hypodermic syringe at an adjustable constant speed. Thus a steady flow of blood to the biceps muscle could be maintained for a desired period of time. The syringe (S) was filled with blood from reservoir A by changing the position of the stopcock at D. While the syringe was being filled, flow to the isolated muscle could be maintained by gravity from reservoir B by changing position of Stopcock E.

Pressure within the system was recorded with a Statham Pressure Transducer, Model P23A (c), which was placed into the perfusion circuit through a T-tube at F. The pressure was recorded through a Grass Bridge

Amplifier on a direct-writing ink oscillograph. Mean pressures were obtained by planimetric integration.

Temperature of the room was recorded continuously on a Brown Recording Thermograph.

Blood samples for analysis of hemoglobin and per cent O_2 saturation were obtained just proximal to the arterial cannula through the three-way stopcock at G.

Radioactive sodium, Na^{22}, 5 microcuries in 0.1 ml. isotonic NaCl, was injected approximately one centimeter into the biceps muscle through a No. 26 gauge needle. Occasionally a small reflux of the injected material would appear at the injection site after the withdrawal of the needle. This was removed with gauze moistened with saline and the surface then blotted with dry gauze.

A lead-shielded Nuclear Scintillation Counter, Model No. DS1 and a Nuclear Scaler, Model No. 165, were used to follow the disappearance rate of the radioactive sodium from the muscle. The scintillation assembly was placed over the injection site one inch from the surface of the muscle. No additional shielding of the aperture of the scintillation tube was used to eliminate beta radiation. (Previously at this distance there was no difference in counting rate between samples counted with an aluminum absorber adequate to eliminate beta radiation and those counted without the aluminum absorber.) Sufficient sample and background counts were measured to assure a standard deviation of ± 2 per cent or less for all but the weakest samples. Rarely, late in an experiment, the one minute counting rate had fallen to a level producing a standard deviation of ± 5 per cent.

After injection of the Na^{22} and placement of the scintillation counter repeated one-minute counts were recorded with fifteen-second intervals between each count. Usually eight successive counts were recorded and the interval of time over which these counts were taken constituted a clearance period. Occasionally as few as five or as many as nine counts were used, depending upon the reproducibility of the volumes of the two-minute collections of venous blood during the clearance period.

Blood flow through the biceps muscle was measured by collecting the venous outflow from the biceps muscle through a cannula in the brachial vein. During all clearance periods the venous blood was collected for two-minute intervals in 10 milliliter volumetric mixing cylinders. Thus the sum of these two-minute volumes was determined during the total time required for a clearance period. The mean venous outflow was then calculated in milliliters per minute. Later when the weight of the biceps muscle was determined, the mean venous outflow was expressed in milliliters per minute per 100 grams of muscle. The sum of the volumes of blood collected for two-minute intervals during a clearance period was determined and the mean flow per two-minute interval was calculated for the entire clearance period. All clearance periods which contained two-minute volumes of blood which deviated more than ten per cent from the mean for that period were discarded. This was taken as evidence of an inconstant flow during the clearance period.

The biceps muscle preparation in this experiment had a completely isolated circulation. Fresh donor dog's blood was infused into the muscular arterial branch supplying the muscle and the total venous outflow from the muscle was collected and discarded. Consequently, in

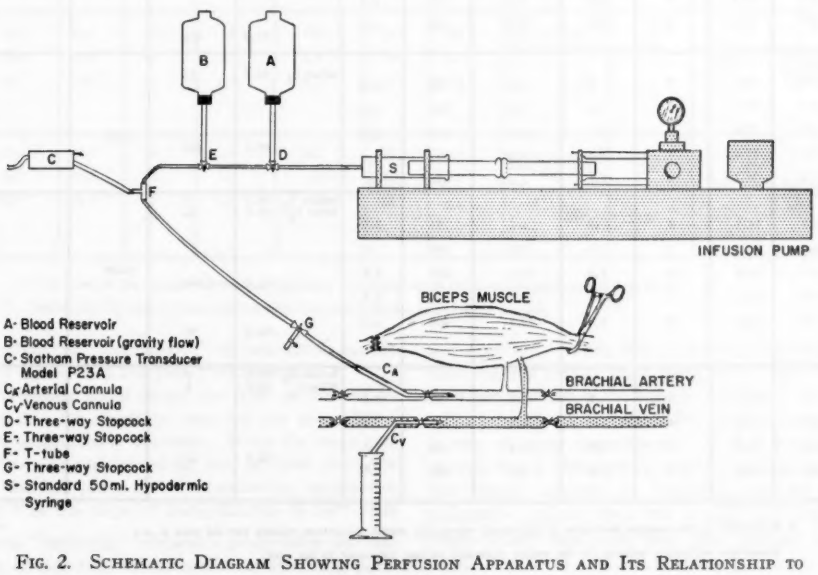


FIG. 2. SCHEMATIC DIAGRAM SHOWING PERFUSION APPARATUS AND ITS RELATIONSHIP TO PERFUSED MUSCLE

TABLE I

Summary of the data from the experiments where Na^{22} was injected intramuscularly—Each horizontal row of figures in a given experiment refers to the data obtained during one clearance period in that experiment

Number of experiment	Clearance rate of Na22 in % per minute	Half-time in minutes	Number of one-minute counts determining clearance rate	Mean biceps venous outflow in ml./minute	Mean biceps venous outflow in ml./min./100 Gm. of muscle	Mean perfusion pressure in mm. of mercury	Maximum deviation of flow from mean flow for entire clearance period in % *	Oxygen content of perfusate in % saturation	Plasma hemoglobin content of perfusate in mg. %	Temperature range of the room in degrees Fahrenheit	Remarks
1	2.2	31.2	7	.7	4.3	100	5.7	94.5	4		Oxygenating device not used.
	3.8	17.9	8	1.1	6.7	135	7.0				
	3.7	18.4	8	2.1	12.8	206	3.0			84-89	
	4.7	14.4	8	2.0	12.2	215	4.0	93.5	4		
	3.1	22.0	8	1.1	6.7	168	2.7				
								89.4	4		
2	2.9	23.6	5	.7	4.0	118	9.0	Before O_2 91.2 After O_2 94.0	10 2		Denervated.
	3.7	18.4	6	1.0	5.7	176	5.2			78-84	
	4.6	14.7	7	1.3	7.4	234	0	92.2	27		
3	2.6	26.3	8	.7	2.7	79	1.5	95.8	11		Oxygenating device not used.
	3.3	20.6	9	1.2	4.7	124	5.5				
	3.2	21.3	8	1.5	5.9	161	8.8			76-82	
	3.9	17.4	8	1.8	7.0	186	7.1	94.8	29		
	2.7	25.3	8	.8	3.1	100	5.2				
	3.7	18.4	7	1.8	7.0	177	3.4				
								93.0	96		
4	1.9	36.1	9	.8	4.0	86	5.0	87.0	3		Oxygenating device not used.
	4.1	16.6	8	1.3	6.5	133	2.3			71-81	
	3.6	18.9	8	1.8	9.0	190	5.7				
	4.6	14.7	8	2.2	11.0	246	3.6				
								87.3	690		
5	2.8	24.4	8	.8	3.2	82	3.8	After O_2 90.6	12		
	4.4	15.4	8	1.3	5.2	117	4.0				
	3.9	17.4	8	1.7	6.8	139	2.3			74-80	
	4.0	17.0	8	2.1	8.4	144	1.4	90.1	400		
	1.9	36.1	6	1.1	4.4	139	0				
6	2.7	25.3	8	.8	4.4	94	3.8	Before O_2 96.4 After O_2 96.9	5 14		
	4.2	16.2	8	1.3	7.2	120	0				
	5.0	13.5	8	1.8	10.0	155	1.6			75-80	
	5.3	12.7	9	2.1	11.7	144	4.2	94.8	60		
	5.3	12.7	9	2.2	12.2	208	3.3				
								94.3	53		
7	2.7	25.3	8	.7	2.6	93	5.4	Before O_2 89.9 After O_2 93.0	2 6		
	4.3	15.8	8	1.2	4.4	136	5.6			80-83	
	4.6	14.7	8	1.7	6.3	165	3.0				
	5.3	12.7	9	1.9	7.0	201	7.6	90.9	46		

* This value is the maximum deviation of individual two-minute venous collection volumes for the mean of all two-minute collection volumes for the entire clearance period, expressed in per cent.

TABLE I—*Continued*

Number of experiment	Clearance rate of Na^{22} in % per minute	Half-time in minutes	Number of one-minute counts determining clearance rate	Mean biceps venous outflow in ml./minute	Mean biceps venous outflow in ml./min./100 Gm. of muscle	Mean perfusion pressure in mm. of mercury	Maximum deviation of flow from mean flow for entire clearance period in % *	Oxygen content of perfusate in % saturation	Plasma hemoglobin content of perfusate in mg. %	Temperature range of the room in degrees Fahrenheit	Remarks
8	3.3	20.6	8	.8	2.6	54	3.8	Before O_2 94.5 After O_2 94.5	2 5	72-77	
	4.9	13.8	8	1.3	4.2	85	9.3				
	6.5	10.3	8	1.7	5.5	116	4.6	96.9	51		
	6.4	10.5	8	2.0	6.5	137	8.3				
9	2.1	32.7	8	.8	3.5	93	6.6	Before O_2 89.1 After O_2 94.8	9 22	75-79	
	3.1	22.0	8	1.2	5.2	130	2.4				
	4.6	14.7	8	1.6	7.0	186	1.8	95.3	192		
	5.4	12.5	7	2.1	9.1	157	6.7				
10	3.7	18.4	9	1.5	5.6	105	9.6	Before O_2 81.0 After O_2 88.8	4 13	74-79	
	3.7	18.4	7	1.4	5.2	117	4.8	89.9	660		
	4.6	14.7	9	1.8	6.7	93	5.5				
	3.5	19.5	9	1.6	5.9	146	4.2	86.8	410		
	4.1	16.6	7	1.8	6.7	89	7.9				
	3.6	18.9	7	1.6	5.9	71	1.8	85.2	222		
					ISCHEMIC AND ANOXIC MUSCLE						
1A	2.6	26.3	8	.8	2.6	56	5.0	Before O_2 84.4 After O_2 94.8	6 45	73-79	Ischemic for 75 minutes prior to injection of Na^{22}.
	3.7	18.4	8	1.4	4.5	59	2.1				
	4.5	15.1	8	1.8	5.8	98	4.3	90.4	415		
	6.2	10.8	8	2.2	7.1	93	3.1				
2A	1.5	45.9	10	.8	2.9	59	6.9	Before O_2 69.4 After O_2 94.8	11 43	73-80	Ischemic for 75 minutes prior to injection of Na^{22}.
	3.8	17.9	8	1.3	4.6	75	3.1				
	5.2	13.0	9	1.6	5.7	114	6.2	97.4	415		
3A	1.4	49.2	8	.8	3.3	54	4.9	Before O_2 64.2 After O_2 90.6	4 11	76-80	Ischemic for 80 minutes prior to injection of Na^{22}.
	2.5	27.4	7	1.2	5.0	84	2.0				
	3.5	19.5	8	1.8	7.5	165	4.4	92.1	158		

* This value is the maximum deviation of individual two-minute venous collection volumes for the mean of all two-minute collection volumes for the entire clearance period, expressed in per cent.

this preparation the "recirculation" of radioactive sodium through the muscle was completely eliminated.

It was frequently noted during the later portions of an experiment that the clearance rate for any given blood flow would progressively decrease. When the same rate of blood flow was maintained for two or three clearance periods in the late phase of the experiments, progressive "flattening" [1] of the slope of disappearance of Na^{22} often was observed. When this "flattening" phenomenon was observed, the results from these latter clearance periods were not included in the data in Table I.

No reinjections of radioactive sodium were performed in any of these experiments. It had previously been learned that a high rate of flow for a long time interval was usually required to completely remove all of the radioactive sodium from the muscle and thus obtain a count which was even near the original background count.

Collateral blood flow, which could have inadvertently

[1] The term "flattening" refers to a progressive decrease in the rate of clearance of radioactive sodium as a function of a given rate of flow.

resulted from failure to ligate small arterial branches and venous tributaries, was checked by placing a hemostat on the perfusion tube which was connected to the arterial cannula. Absence of venous outflow during this procedure was accepted as evidence of an isolated circulation to the biceps muscle. Either small arterial branches joining the brachial artery distal to the cannula or small venous tributaries joining the brachial vein proximal to the venous cannula would have resulted in a flow of blood from the venous cannula. If evidence for collateral vessels was found, and the vessels could not be obliterated, the experimental data were considered invalid and not reported.

At the termination of each experiment India Ink was injected into the arterial cannula. The ink flowed relatively easily through the muscle vasculature and drained from the venous cannula. No evident swelling of the muscle was produced by perfusion of either the blood or the colloidal suspension of India Ink. The biceps muscle was then removed and sectioned with a scalpel. In each experiment the muscle was found to be diffusely and homogeneously darkened. The muscle was then weighed. Section of neighboring muscles in the foreleg with a scalpel revealed no evidence of darkening with the colloidal suspension.

Experiment Nos. 1A, 2A, and 3A were performed in the usual way except that the muscle was purposely made anoxic and ischemic. After cannulation of the vessels, and prior to the onset of perfusion and injection of the radioactive sodium intramuscularly, the muscle was allowed to remain without any blood supply for a period of 75 to 80 minutes. Then the perfusion and injection of the Na^{22} were carried out in the usual way.

Experiment Nos. 1B, 2B, and 3B were performed to

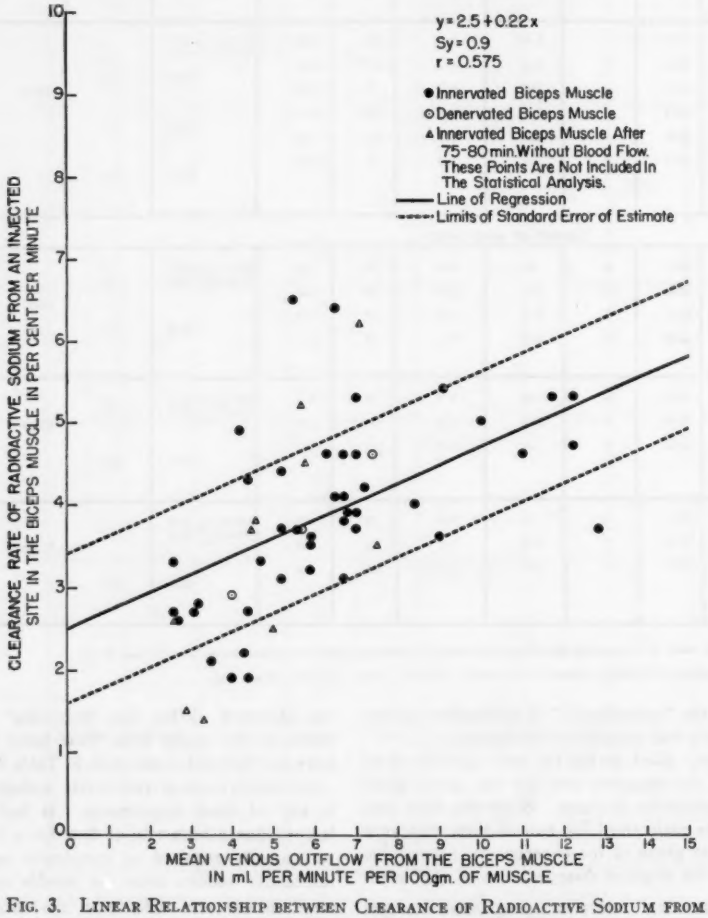


FIG. 3. LINEAR RELATIONSHIP BETWEEN CLEARANCE OF RADIOACTIVE SODIUM FROM INJECTED SITE IN MUSCLE AND BLOOD FLOW THROUGH THE MUSCLE

The wide variation in clearance rate for any given flow is evident.

test the closeness of correlation between flow rate and clearance of Na^{22} from muscle following its injection into the arterial supply to that muscle. They differed from the other experiments in the following respects. Instead of injecting the radioactive sodium intramuscularly, Na^{22} (25 microcuries in 0.5 ml. in Experiment Nos. 1B and 2B and 20 microcuries in 0.4 ml. in Experiment No. 3B) was injected into the perfusion tube just proximal to the intra-arterial cannula. In Experiment Nos. 1B and 2B the clearance periods were much longer than in previous experiments. In Experiment No. 3B the clearance periods were of the usual length. At the termination of each of these experiments the biceps muscle was injected with India ink and then removed without changing the original position of the scintillation tube. In each instance the counts after removal of the muscle were very close to background and it was concluded that there was very little contamination of the tubing and cannulae distal to the injection site.

The oxygen saturation of the blood used for perfusion was determined by the method of Gordy and Drabkin (6).

The plasma hemoglobin concentration of the blood used for perfusion was determined by the method of Creditor (7).

RESULTS

Relationship between blood flow and clearance rate of radioactive sodium from an intramuscular injection site in muscle with an adequate supply of oxygenated blood

The over-all results of the correlation between the clearance rate of radioactive sodium from an injected site in the biceps muscle and the blood flow through the muscle can be seen in Table I and Figure 3. A linear relationship as determined statistically was noted between clearance and flow. However, the large standard error of estimate about the line of regression prevented accurate estimation of flow from a given clearance value.

The linearity of the line of regression was tested by the analysis of variance about the line using the method of Fisher (8). The deviations from linear regression were not significant—they were fully accounted for by the variations within the arrays; so linearity was indicated. The equation found for the line of regression was $y = 2.5 + .22 \times$. The standard error of estimate about this line of regression was 0.9. Thus 99 per cent of the clearance values for a given flow would fall between plus or minus 2.7 per cent (2.7 per cent referring to units of the ordinate rather than per cent variation about the line). The coefficient of correlation was 0.57. This can be considered a

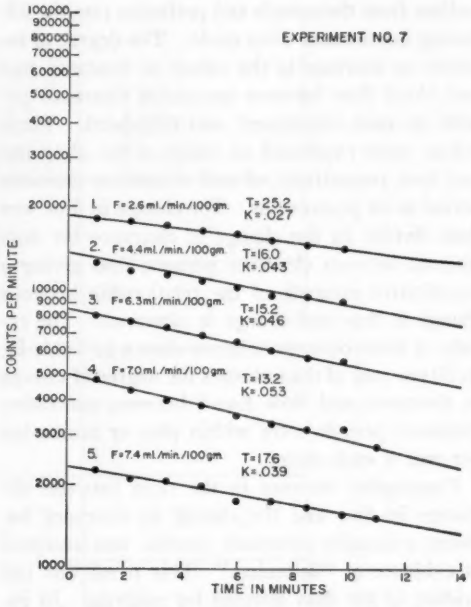

EXPERIMENT NO. 7
COUNTS PER MINUTE
TIME IN MINUTES
1. F=2.6 ml./min./100 gm. T=25.2 K=.027
2. F=4.4 ml./min./100 gm. T=16.0 K=.043
3. F=6.3 ml./min./100 gm. T=15.2 K=.046
4. F=7.0 ml./min./100 gm. T=13.2 K=.053
5. F=7.4 ml./min./100 gm. T=17.6 K=.039

FIG. 4. PROGRESSIVE INCREASE IN EXPONENTIAL SLOPES OF DISAPPEARANCE OF NA^{22} FROM INJECTED SITE IN MUSCLE AS A FUNCTION OF INCREASING PERFUSION RATES

Slope 5 illustrates the "flattening" effect seen in later phases of some experiments.

significant correlation since the value of "z", derived from the coefficient of correlation, exceeded three times the standard error of "z".

Figure 4 demonstrates typical slopes of disappearance of radioactive sodium from an injected site in the biceps muscle with increasing rates of perfusion during successive clearance periods. (Taken from Experiment No. 7.) It can be seen that clearance rate and blood flow rise concordantly during the first four clearance periods. However, progressive decrease in clearance rate, as a function of flow, was observed during the last two clearance periods even though the flow was maintained relatively constant. Data from the fifth and sixth clearance periods of this experiment were not included in the analysis of the results. Similar terminal clearance periods from those other experiments which demonstrated "flattening" were likewise not included.

To further clarify, in a quantitative way, the relationship between clearance rate of radioactive

sodium from the muscle and perfusion rate the following calculations were made. The degree of increase or decrease in the values of clearance rate and blood flow between successive clearance periods in each experiment was calculated. These values were expressed as ratios of the clearance and flow, respectively, of each successive clearance period to its predecessor. The change in flow was then divided by the change in clearance for each interval between clearance periods, thus giving a quantitative estimate of the relationship between change in flow and change in clearance. The results of these computations are shown in Table II. In 50 per cent of the instances the degree of change in clearance and flow found between successive clearance periods were within plus or minus ten per cent of each other.

Progressive increase in the ratio between the change in flow and the change in clearance between successive clearance periods was accepted as evidence of "flattening." This trend was not evident in the data selected for analysis. In instances where it was noted, invariably in the later clearance periods of an experiment, these data were deleted.

The flow produced by the infusion pump was pulsatile in character having about 40 beats per minute. The pulse pressure varied between 8 and 18 millimeters of mercury, independent of the total pressure within the system.

There was no obvious relationship noted between the ambient temperature and the results of any of the experiments.

In view of the previous reports that elevated plasma hemoglobin produces vasodilatation, the hemoglobin concentration in the perfusating plasma was measured. No relationship was found between levels of plasma hemoglobin and the results of the experiments.

Relationship between blood flow and clearance rate of radioactive sodium from an intramuscular injection site in muscle following a 75 to 80-minute period of ischemia and anoxia

The data from three experiments in which radioactive sodium was injected into an ischemic

TABLE II

Table of ratios indicating the quantitative changes in clearance rates (C) and flow rates (F) during successive clearance periods, and the relationship of change in flow rate to change in clearance rate

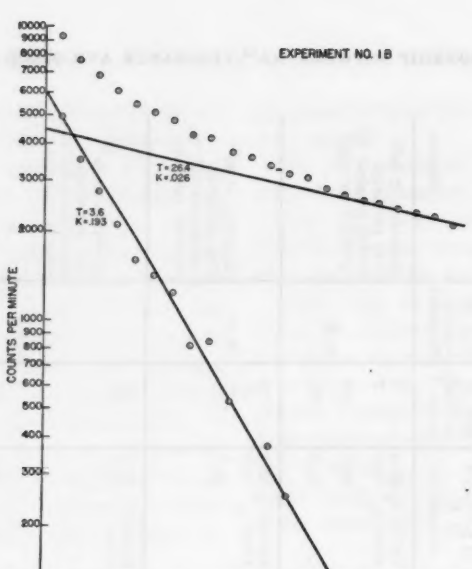
Number of experiment	Clearance periods determining ratio	Ratio C_2/C_1	Ratio F_2/F_1	$\frac{F_2}{F_1} \div \frac{C_2}{C_1}$
1	2/1	1.73	1.56	.90
	3/2	.97	1.91	1.96
	4/3	1.27	.95	.75
	5/4	.66	.55	.83
2	2/1	1.27	1.42	1.12
	3/2	1.24	1.30	1.05
3	2/1	1.27	1.74	1.37
	3/2	.97	1.25	1.28
	4/3	1.22	1.19	.97
	5/4	.69	.44	.64
	6/5	1.37	2.26	1.65
4	2/1	2.16	1.62	.75
	3/2	.88	1.38	1.56
	4/3	1.28	1.22	.95
5	2/1	1.57	1.62	1.03
	3/2	.89	1.31	1.47
	4/3	1.02	1.23	1.20
	5/4	.47	.52	1.11
6	2/1	1.55	1.64	1.06
	3/2	1.19	1.38	1.16
	4/3	1.06	1.17	1.10
	5/4	1.00	1.04	1.04
7	2/1	1.60	1.69	1.06
	3/2	1.07	1.43	1.34
	4/3	1.15	1.11	.96
8	2/1	1.48	1.61	1.09
	3/2	1.33	1.31	.98
	4/3	.98	1.18	1.2
9	2/1	1.48	1.48	1.0
	3/2	1.48	1.35	.91
	4/3	1.17	1.30	1.11
10	2/1	1.00	.93	.93
	3/2	1.24	1.29	1.04
	4/3	.76	.88	1.16
	5/4	1.17	1.13	.96
	6/5	.88	.88	1.00
	Ischemic and Anoxic muscle			
1A	2/1	1.42	1.73	1.22
	3/2	1.22	1.28	1.05
	4/3	1.38	1.22	.88
2A	2/1	2.53	1.59	.63
	3/2	1.37	1.24	.91
3A	2/1	1.78	1.51	.85
	3/2	1.40	1.50	1.07

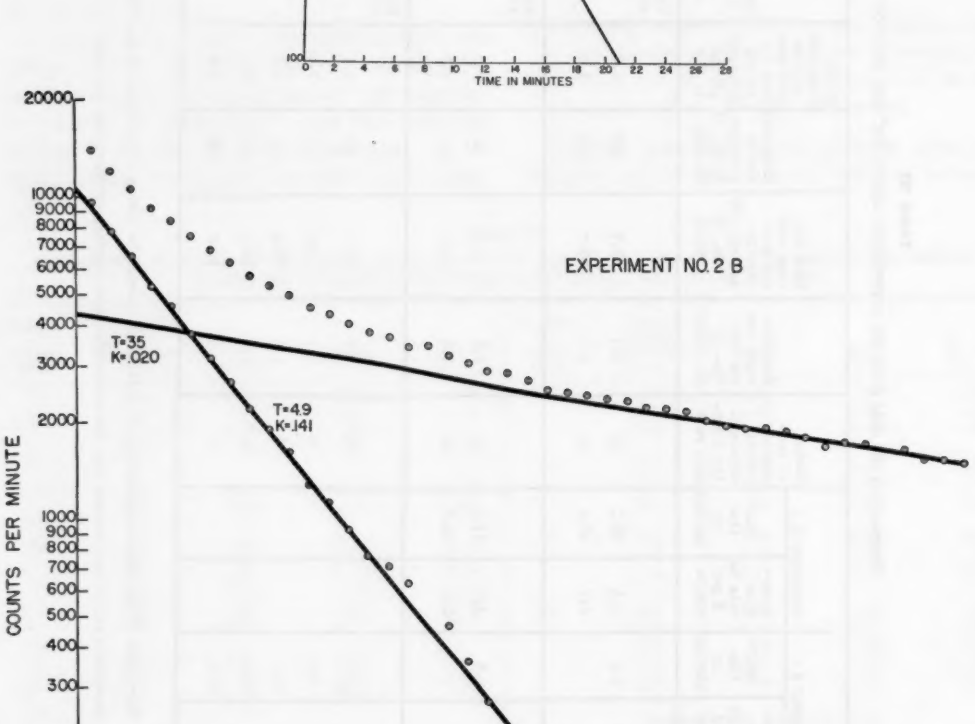
TABLE III

Summary of the data from the experiments where Na^{22} was injected into arterial supply

Number of experiment	COMPONENT # 1		COMPONENT # 2		Number of one-minute counts determining clearance rate	Mean biceps venous outflow in ml./minute	Mean biceps venous outflow in ml./min./100 Gm. of muscle	Mean perfusion pressure in mm. of mercury	Maximum deviation of flow from mean flow for entire clearance period in % *	Oxygen content of perfusate in % saturation	Plasma hemoglobin content of perfusate in mg. %	Temperature range of the room in degrees Fahrenheit	Remarks
	Clearance rate in % per minute	Half-time in minutes	Clearance rate in % per minute	Half-time in minutes									
1B										Before O_2 88.6	2		Injection of Na^{22} into perfusion tubing proximal to arterial cannula.
										After O_2 93.0	5		
	19.3	3.2	2.6	26.3	23	1.3	5.9	130	4.3				
										95.1	900	74-82	
			2.7	25.3	11	2.1	9.5	154	4.5				
										93.0	270		
2B										Before O_2 80.5	20		
										After O_2 94.0	28		
	14.1	4.6	2.0	34.3	45	1.3	3.7	81	7.7		1600	78-82	Injection of Na^{22} into perfusion tubing proximal to arterial cannula.
										92.5			
			1.3	53.0	11	2.0	5.7	113	3.7				
3B										Before O_2 58.4	10		
										After O_2 96.9	23		
	5.5	12.3			5	.7	3.7	62	5.3				Injection of Na^{22} into perfusion tubing proximal to arterial cannula.
	8.7	7.6			6	1.1	5.8	105	3.6				
	5.2	13.0			6	1.6	8.4	132	4.1			74-78	
										96.1	405		
	3.9	17.4			6	1.9	10.0	159	8.5				
	2.7	25.3			6	1.9	10.0	198	3.2				
										90.9	49		

* This value is the maximum deviation of individual two-minute venous collection volumes from the mean of all two-minute collection volumes for the entire clearance period, expressed in per cent.





FIGS. 5 AND 6. TWO COMPONENT EXPONENTIAL SLOPE OF DISAPPEARANCE OF Na^{22} FROM PERFUSED MUSCLE AFTER ITS INJECTION INTO THE ARTERIAL SUPPLY TO THE MUSCLE

and anoxic muscle are recorded in the latter part of Table I. In each of these experiments the muscle was completely without blood flow for a period of 75 to 80 minutes. The relationship between clearance rate of radioactive sodium and rate of blood flow in this preparation, following this period of ischemia, was very similar to that found in the first ten experiments in which the muscle had an adequate supply of oxygenated blood (Figure 3). During the first clearance period of Experiment Nos. 2A and 3A, during which the rate of blood flow was low, the clearance rate of radioactive sodium was less than that seen in any oxygenated muscle for a similar range of blood flow. However, the subsequent clearance values for any given blood flow for these experiments, as well as all the clearance values in Experiment No. 1A, fell within the scatter of data for the first ten experiments. The data from these three experiments are not included in the statistical analysis of the data for the first ten experiments.

Relationship between blood flow and clearance rate of radioactive sodium from muscle following intra-arterial injection of Na^{22} into the nutrient artery supplying the muscle

Three experiments were performed in which the clearance rate of radioactive sodium from the biceps muscle was determined following the injection of Na^{22} into the perfusion tube immediately proximal to the arterial cannula (Table III). In two of these experiments (1B and 2B) an attempt was made to maintain the perfusion rate at a constant level until the clearance rate of Na^{22} from the muscle had reached a constant value. In Experiment No. 2B it was necessary after the first thirty minutes, during which time the flow was maintained constant at a level of 4.0 ml. per minute per 100 Gm. of muscle (maximum deviation 4.4 per cent) to refill the syringe on the pump. After this was accomplished the flow was maintained at a slightly lower level, 3.4 ml. per minute per 100 Gm. of muscle (maximum deviation 9.8 per cent). The values recorded for the first clearance period in the data in Table III are the average of these two values. The maximum deviation from mean two-minute flow was calculated using the mean of all two-minute collection volumes throughout the entire 53.5 minutes. In both experiments the disappearance rate of radioactive sodium from the muscle could be separated into two exponential components with widely variant rate constants. The results are illustrated in Figures 5 and 6. In both of these experiments, when a constant rate of clearance appeared to have been reached, the perfusion rate was raised. In Experiment No. 1B the perfusion rate was raised from 5.9 to 9.5 ml. per min. per 100 Gm. and in Experiment No. 2B from 3.7 to 5.7 ml. per min. per 100 Gm. In neither instance was there any resultant increase in the clearance rate subsequent to the increase in perfusion rate. In 2B the clearance rate during this period of increased flow actually decreased slightly. The values for the last clearance periods in 1B and 2B were recorded under "Component No. 2" in Table III since they differed little from the slower component.

In Experiment No. 3B perfusion was started at a low rate, 3.7 ml. per min. per 100 Gm., and was increased stepwise during four subsequent clearance periods to a maximum of 10 ml. per min. per 100 Gm. Table III shows the results. There was a rise in the clearance rate during the second clearance period as compared to the first, concomitant with the rise in perfusion rate. However, during successive clearance periods thereafter the clearance rates decreased progressively in spite of increasing perfusion rates. During the last two clearance periods a relatively constant perfusion rate of 10 ml. per min. per 100 Gm. was maintained and the clearance rates continued to decrease, going from 3.9 per cent in the fourth clearance period to 2.7 per cent in the last.

DISCUSSION

The results of the intramuscular injection experiments, namely, the wide range of blood flow represented by any given clearance rate of radioactive sodium, tend to minimize the value of this method as an index of total muscle blood flow.

There are several factors which may be responsible in greater or lesser degree for this lack of correlation between rate of clearance of radioactive sodium from an intramuscular injection site and total blood flow through the muscle. These can be divided conveniently into physiological factors and anatomical factors.

Physiological factors

The presence of effective shunts at the arteriolar level which by-pass a fraction of the arterial blood around the capillary circulation could be important in producing the observed lack of correlation between total blood flow and radioactive sodium clearance. Since, presumably, radioactive sodium is cleared from the injected site only by blood flow at the capillary level, the radioactive sodium clearance would correlate only with that fraction of total blood flow not shunted. What quantitative significance shunts actually possess is not known at the present time and therefore their influence on the present data cannot be evaluated.

Pappenheimer, Renkin, and Borrero (9) have shown from their data on osmotic transients that at low blood flows the diffusion rate of sodium chloride is flow limited. The question arises as to whether, at higher levels of blood flow, the exchange of sodium between the vascular system and extracellular space may be limited by permeability factors and the diffusion rate of sodium rather than by blood flow *per se*. Jones (10) presented evidence that the exchange rates in various tissues of several inert gases, having an eightfold range of relative diffusion rates, were approximately the same. These results led to the view that diffusion and transcapillaric permeability were not factors limiting the inert-gas-exchange rate and that in fact blood flow determined the exchange rate. The linearity of our clearance/flow relationship and the absence of any consistent reduction in increment of clearance rates at higher blood flows suggest that radioactive sodium exchange in these experiments was not limited at higher levels of blood flow by diffusion or permeability factors.

Although sodium is freely diffusible it is certainly not inert and consequently is subject to such influences as electric potential gradient and chemical combination. What effect these influences had on our results cannot be assessed.

It is now known that muscle fibers are not impermeable to sodium and hence that an energy-using transport system must be operative to drive outward the sodium that enters by diffusion and thus to keep it in its primarily extracellular location (11, 12). That this "sodium pump" may have been injured via anoxia and other factors during the dissection was a possibility. How such injury would effect our clearance values was not known. However, it was suspected that by allowing a portion of radioactive sodium to diffuse into the muscle fibers the amount available for clearance might have been decreased. The three anoxic experiments were carried out to measure the effects of complete absence of blood supply over a 75 to 80-minute interval on the clearance of radioactive sodium. The data from these experiments did not indicate any obvious effect of anoxia on the clearance rate of radioactive sodium as a function of flow. In light of these findings, as well as the fact that we were perfusing with relatively well-oxygenated blood, it would appear that anoxia was not a significant contributing factor to the wide scatter of data.

Anatomical factors

The anatomical factors which could possibly affect the correlation between clearance rate and flow have been discussed by others. They involve principally the vascularity of the site selected for injection, the degree of tissue injury produced by the injection, and the amount of distortion and compression of local tissues produced by the injected fluid. It has been shown recently (13) that the clearance rate is dependent upon the volume of fluid injected into the muscle; larger volumes result in lower clearances. The present studies shed no further light on this general aspect of the problem but it seems likely that these local anatomical factors may be of importance in producing some of the discrepancies in correlation.

The clearance rate following intra-arterial injection of radioactive sodium was measured in three experiments to observe its correlation with blood flow. By injecting radioactive sodium ion into an artery, a large portion of the injected material can be localized in the region supplied by the vessel due to the rapid exchange which occurs with the local sodium pool. The subsequent rate of removal (clearance) of this residual material can then be measured. Dobson, Warner, Pace, Finney, and Johnston (14) reported the original studies using this method and found that "the rate of removal is not constant and is much more rapid than it is in the case of local injections. The variation of the removal rate with time can

be explained on the basis of differing degrees of vascularity in the tissue."

If the diffusion rate of sodium is very rapid over such tissue and cell barriers as may exist, compared to the rate of movement of blood through the capillaries, then the clearance rates following intra-arterial injection should reflect vascularity of tissue. The quantitative aspects of blood flow as determined by clearance rates of intra-arterially injected Na^{22} could be handled analogously to the methods of Jones (10) using inert gases except for two correction factors to allow for 1) the differing concentration of sodium between blood and tissue and 2) the fact that sodium is limited to the sodium space. The above interpretation would lead one to believe that in Experiments 1B and 2B there were two differently vascularized areas of tissue within the muscle; the one with a high time constant having a relatively high rate of flow and the one with a low time constant having a relatively low rate of flow for a given total blood flow to the muscle. One would have expected, however, that in Experiments 1B and 2B an increase in the remaining slow component should have occurred subsequent to an increase in total blood flow. That this did not occur would tend to invalidate the conclusion that the clearance rates of sodium from muscle following intra-arterial injection were dependent only upon blood flow to the part.

In Experiment 3B, except for the second clearance period, the clearance rates decreased progressively in spite of increasing blood flow. Interpretation here is more difficult. These results could occur 1) because clearance rate was dependent upon other factors as well as blood flow, and 2) that during clearance periods 1 and 2 most of the radioactive sodium was removed from the more highly vascularized area and subsequent decreases in clearance were accounted for by increasing predominance of clearance from the less vascularized area.

SUMMARY AND CONCLUSIONS

Ten experiments were performed to establish the correlation between the clearance rate of radioactive sodium from an intramuscular injection site and total blood flow through the biceps muscle of the dog. A linear relationship was found to exist between these two variables. However, the scatter of data around the line of regression was so great that the clearance rate of radioactive sodium could not be utilized as an accurate measure of total blood flow.

Three similar experiments were performed after a 75 to 80-minute interval during which time the biceps muscle was completely deprived of blood supply. Comparison of results from these three experiments with those of the previous ten revealed no obvious difference in the relationship between clearance and flow. These results, coupled with the fact that the muscle was perfused with relatively well oxygenated blood, were considered as evidence that anoxia was not a significant contributing factor to the wide scatter of the data in the first ten experiments.

Three experiments were performed in an attempt to establish the correlation between the clearance rate of radioactive sodium from muscle, subsequent to its intra-arterial injection, and blood flow through the muscle. The data from these experiments did not support the concept that the clearance rate of radioactive sodium under these circumstances was solely dependent upon blood flow.

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THE EFFECT OF PROTOVERATRINE ON THE CARDIAC OUTPUT AND ON SOME REGIONAL CIRCULATIONS IN MAN[1]

By S. W. HOOBLER, T. G. KABZA, AND R. W. CORLEY[2]

(From the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich.)

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Protoveratrine is a mixture of two purified alkaloids derived from *veratrum album*. According to Meilman and Krayer (1) it has a marked and reproducible depressor effect when given intravenously to hypertensive patients. This effect has been confirmed (2) and the drug has been administered in the long term management of severe hypertension (3). It therefore becomes important to know whether the depressor action of Protoveratrine[3] is a result of a reduction in cardiac output, or of a decrease in total peripheral resistance, and if the latter, what vascular area is chiefly involved. Observations bearing on these questions are presented herewith.

METHODS

The Protoveratrine was prepared for intravenous administration according to the method of Meilman and Krayer (1). Subjects were patients with essential or renal hypertension under the age of 50 and with casual diastolic blood pressures exceeding 110 mm. of mercury. Except where indicated, they had not undergone supradiaphragmatic sympathectomy. The cardiac output was determined by the direct Fick method, the mixed venous sample being obtained in all instances from the pulmonary artery. Blood gas analyses were determined by the Hickam and Frayser technique (4) and expired air was collected over 1- to 2-minute periods in a spirometer and measured by a Beckman oxygen analyzer. Renal studies were performed by the usual clearance techniques, utilizing para-amino-hippurate and mannitol (5, 6). Peripheral blood flow was measured by the venous occlusion plethysmograph following the technique outlined by Abramson (7).

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[2] Present address is 204 Holmcrest Street, Jackson, Michigan.

[3] Kindly supplied to us at first by Dr. Otto Krayer, Professor of Pharmacology, Harvard University Medical School, and later by Dr. Kenneth Kohlstaedt, Eli Lilly and Company.

RESULTS

Effects on blood pressure and pulse rate

Preliminary experience with Protoveratrine indicated that when an effective dose (1.2 to 1.6 mcg. per Kg.) was administered intravenously in single injections, a 10 to 20 per cent reduction in mean blood pressure usually occurred in the hypertensive patients. The lowering of pressure was associated with a marked bradycardia, which could be abolished by atropine sulphate 1 mg. intravenously. The blood pressure remained unchanged in some cases while in other instances it rose considerably following the atropine. No cases of heart block or cardiac irregularity were encountered after Protoveratrine. The reduction in blood pressure began in 5 to 10 minutes, reached a maximum in 7 to 15 minutes and gradually returned toward the initial control levels in the remaining 30 to 45 minutes. The patients experienced a few side effects, chief of which were paresthesias in the throat, over the face and upper thorax. Nausea and vomiting were not observed after single intravenous injections.

Effect of the drug on cardiac output and peripheral resistance (Table I).

In all cases there was a decline in cardiac output at the time of the lowest blood pressure, although in a few cases the blood pressure decline preceded the fall in output (E. B., S. P.). The data are presented in Table I for those patients who had a fall greater than 10 per cent in their mean blood pressures following injection of Protoveratrine. Three patients whose blood pressure did not fall were excluded from the table, since no significant alterations in the pulse rate or cardiac output occurred. The decline in cardiac output was associated with a decrease in heart rate, so that stroke volume usually remained unchanged.

TABLE I

Effect of intravenous Protoveratrine on cardiac output in hypertensive subjects

Subj.	Time (*Min.*)	Pulse rate	Blood press. (*mm. Hg*) S/D	Mean	% Δ of mean BP	A-V O_2 diff. (*Vol. %*)	Cardiac index *L./min./m.²*	% Δ	Stroke vol. per. resist. *cc.*	% Δ	*dynes/sec./cm.⁻⁵*	% Δ
E. B.	−14	86	180/130	155		4.8	3.16		68		1,135	
	− 7	84	187/128	158		4.7	3.14		70		1,165	
	I.V. PV (.14 mg.)											
	+ 6	74	135/88	112	−28	4.4	3.42	+ 9	86	+25	755	−34
	+11	70	122/80	101	−36	5.1	2.60	−17	69	0	900	−22
	+18	72	142/95	118	−25	5.0	2.75	−13	71	+ 3	990	−14
	+37	78	162/118	140	−10	4.7	3.02	− 4	72	+ 4	1,070	− 7
	+56	84	165/120	142	− 9	4.7	3.05	− 3	68	− 1	1,075	− 7
E. Br.	−20	78	225/140	182		4.5	2.61		50		2,475	
	− 7	71	240/140	190		4.3	2.79		59		2,415	
	I.V. PV (.09 mg.)											
	+11	52	145/100	122	−34	4.8	2.11	−22	61	+13	2,050	−16
	+20	51	130/95	112	−40	5.2	1.93	−28	57	+ 6	2,065	−16
S. P.	−13	80	254/140	197		5.3	3.00		63		1,855	
	− 5	80	245/134	190		5.1	3.03		64		1,775	
	I.V. PV (.13 mg.)											
	+10	66	197/99	148	−24	4.7	3.14	+ 4	80	+25	1,335	−26
	+18	63	170/96	133	−31	5.5	2.30	−24	61	− 5	1,640	−10
	+29	65	190/107	148	−24	5.4	2.52	−17	65	+ 2	1,660	− 8
	+48	74	224/126	175	−10	5.2	2.59	−14	59	− 8	1,915	+ 6
J. L.	−12	87	210/160	185		4.1	3.48		68		1,490	
	− 4	90	210/160	185		4.0	3.60		68		1,445	
	I.V. PV (.12 mg.)											
	+19	80	180/140	160	−14	4.8	3.08	−13	65	− 4	1,440	− 2
	+27	79	175/140	158	−15	4.6	3.07	−13	66	− 3	1,440	− 2
C. K.	−11	74	195/132	164		4.7	3.63		82		1,295	
	− 4	74	198/135	166		4.6	3.44		78		1,385	
	I.V. PV (.11 mg.)											
	+21	66	172/125	148	−10	4.7	3.01	−15	76	− 5	1,410	+ 5
	+32	69	182/130	156	− 5	4.7	3.21	− 9	78	− 2	1,395	+ 4
	+38	71	188/132	160	− 3	4.5	3.41	− 4	80	0	1,345	0
S. F.	−17	73	171/119	145		4.8	2.80		64		1,485	
	− 7	76	175/118	146		4.7	2.83		62		1,475	
	I.V. PV (.12 mg.)											
	+ 5	74	138/107	122	−16	5.0	2.57	− 9	58	− 8	1,360	− 8
	+10	69	122/97	110	−24	5.6	2.24	−21	54	−14	1,410	− 5
	+30	69	145/103	124	−15	5.1	2.58	− 8	62	− 2	1,380	− 7
	+48	74	153/105	129	−11	5.7	2.32	−18	52	−17	1,590	+ 7

In the first three patients in whom the blood pressure was markedly reduced, a decrease in total systemic peripheral resistance also occurred. In the remainder, the percentage of reduction in blood pressure was similar to the reduction in cardiac output, so that the total peripheral resistance did not change.

In order to study the effects of a more gradual reduction in blood pressure, the cardiac output was followed for three hours in one patient given the drug orally (Figure 1). In this case, reduction of pressure was not associated with a decline in cardiac output and there was, consequently, a marked fall in total peripheral resistance.

Cardiac work (calculated as the product of cardiac output and mean blood pressure) was reduced in all cases given Protoveratrine.

Effects on renal circulation

Six of ten patients studied showed reductions exceeding 10 per cent in mean blood pressure after intravenous Protoveratrine (Table II). In these cases no consistent changes in renal plasma flow occurred. Consequently, a slight reduction in renal resistance was observed. Glomerular filtration rate fell more than 10 per cent in four of the ten cases, and the filtration fraction was correspondingly lower. The most striking altera-

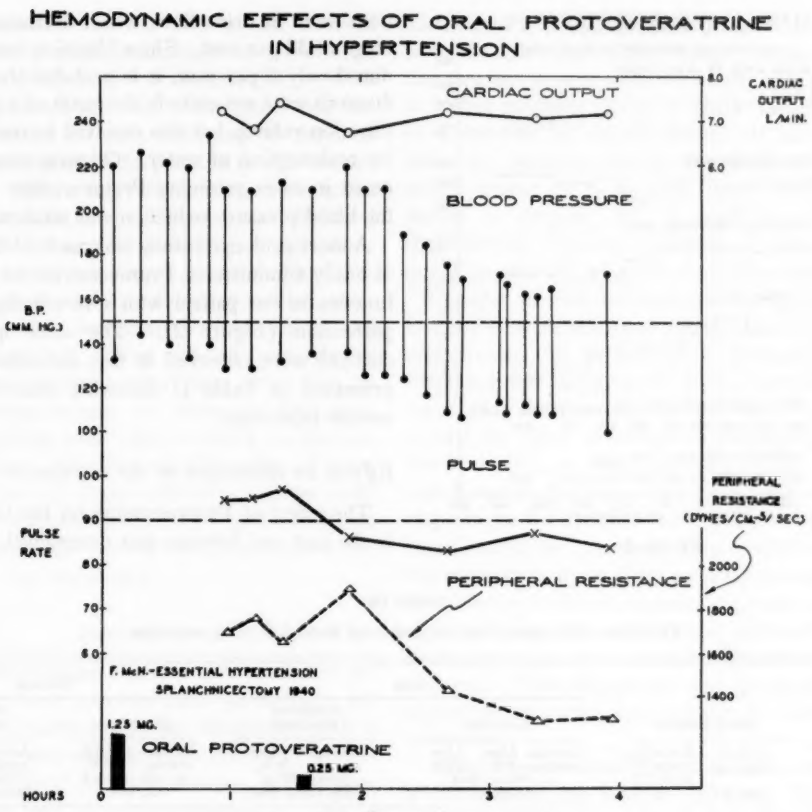

FIGURE 1

TABLE II

The effects of Protoveratrine on renal circulation in hypertension *

Subj.	Mean blood pressure: Control *Mm. Hg*	Mean blood pressure: After P.V. % Δ	Renal plasma flow: Control *cc./min.*	Renal plasma flow: After P.V. % Δ	Renal resistance: Control Mean BP RPF	Renal resistance: After P.V. % Δ	Glomerular filtration rate: Control *cc./min.*	Glomerular filtration rate: After P.V. % Δ	Filtration fraction: Control	Filtration fraction: After P.V. % Δ	Urine flow: Control *cc./min.*	Urine flow: After P.V. % Δ
H. S.	149	−18	513	+ 2	.29	−19	118	− 6	.23	− 9	4.2	0
P. J.	159	−16	180	+16	.88	−24	55	+11	.31	− 6	2.4	+ 8
L. L.	223	−14	403	−11	.55	− 3	104	−16	.26	− 8	3.3	−58
M. B.	145	−14	525	+ 3	.27	−16	108	−12	.21	−14	4.7	−43
B. L.	146	−13	370	+ 2	.39	−14	90	− 6	.24	− 4	3.1	−29
M. A.	157	−11	359	− 3	.43	− 8	97	−12	.26	− 8	4.8	−60
F. C.	182	− 8	274	−11	.66	3	63	−13	.23	− 4	7.8	−63
M. D.	188	− 6	306	− 4	.61	− 1	95	− 6	.31	− 3	3.8	−34
M. W.	167	− 5	466	+11	.35	−14	115	+ 3	.25	− 8	8.9	−58
C. F.	162	− 3	299	− 7	.54	3	73	− 1	.24	8	4.6	−43
Mean Value	168		370		.50		92		.25		4.8	
% Change		−11		0		− 9		− 6		− 6		−38

* All control observations represent the mean of three ten-minute periods immediately preceding the intravenous injection of Protoveratrine. Data listed as "after Protoveratrine" represent the mean value of two or three successive urine collection periods starting ten minutes after intravenous Protoveratrine and running continuously up to 30 to 40 minutes after the injection, depending on the duration of significant BP reduction. Renal plasma flow was determined by para-amino-hippurate clearance and glomerular filtration rate by mannitol clearance.

P.V. = Protoveratrine.

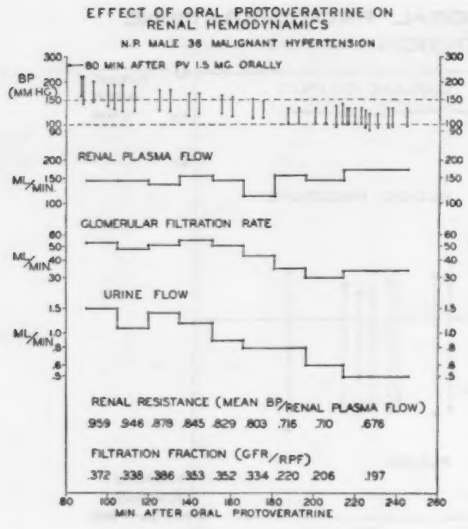
EFFECT OF ORAL PROTOVERATRINE ON RENAL HEMODYNAMICS
N.P. MALE 36 MALIGNANT HYPERTENSION
80 MIN. AFTER PV 1.5 MG. ORALLY
BP (MM HG)
RENAL PLASMA FLOW
ML/MIN.
GLOMERULAR FILTRATION RATE
ML/MIN.
URINE FLOW
ML/MIN.
RENAL RESISTANCE (MEAN BP/RENAL PLASMA FLOW)
.959 .946 .878 .845 .829 .803 .715 .710 .676
FILTRATION FRACTION (GFR/RPF)
.372 .338 .386 .353 .352 .334 .220 .206 .197
MIN. AFTER ORAL PROTOVERATRINE

FIGURE 2

tions were in urine flow, which decreased an average of 38 per cent. Since filtration rate was reduced only 6 per cent, it is probable that the reductions were not entirely the result of a decreased filtration volume, but also involved increased tubular reabsorption of water. Oliguria was likewise noted in cases receiving Protoveratrine in whom the blood pressure reduction was minimal.

A more prolonged study was made of the effects of orally administered Protoveratrine on the renal function in one patient with severe malignant hypertension (Figure 2). The same qualitative changes were observed in this individual as are presented in Table II following effective intravenous injections.

Effects on circulation in the extremities

The effect of Protoveratrine on the blood flow in the foot and forearm was determined in 21 re-

TABLE III

The effects of Protoveratrine on peripheral blood flow in hypertension

			Foot					Forearm			
	Blood pressure		Blood flow			Peripheral resistance		Blood flow		Peripheral resistance	
Name	Systolic / Diastolic *mm. Hg*	Reduction mean BP after P.V. %	Average resting *cc./100 cc. limb vol./min.*	After P.V.	After TEA	Average resting (Mean BP ÷ Mean blood flow)	After P.V.	Average resting *cc./100 cc. limb vol./min.*	After P.V.	Average resting (Mean BP ÷ Mean blood flow)	After P.V.
W. B.	194/138	−43	3.1	6.9		53.5	13.7				
H. S.	155/114	−33	2.2	3.1	5.0	61	29				
E. S.	220/128	−30	3.4	5.7		51	21				
J. C.	190/134	−29	2.1	3.0		77	38				
T. L.	204/126	−28	3.3	4.0		50	29	5.3	6.6	31	18
M. J.	199/116	−24	5.9	8.5		27	14				
E. G.	175/128	−23	3.2	7.0	8.8	47	16.7				
E. S.	188/116	−21	.7	1.8	4.2*	217	67				
L. C.	186/142	−21	1.7	6.2	7.8	96	21				
J. P.	207/145	−19	3.3	5.4		53	26	3.4	5.7	52	25
L. L.	174/116	−18						2.7	3.9	54	30
W. M.	210/142	−15	5.8	5.4		30	28	6.2	5.4	28	27
M. A.	200/140	−15	1.7	2.5	4.9*	100	58				
M. D.	208/134	−14	1.8	2.4		95	61	.6	.6	285	245
E. S.	189/129	−14	3.9	5.0		41	27	6.0	6.7	26	20
H. R.	189/125	−13						3.3	5.1	48	27
D. G.	184/100	− 9						2.2	4.4	65	29
M. S.	148/110	− 9						5.0	8.9	26	13
Mean, all hypertensives		−21	*3.1*	*4.8*		*71*	*32*	*3.8*	*5.25*	*68*	*48*
Postop. lumbar sympathectomy											
P. G.	204/114	− 3	4.2	3.1		35	50				
I. K.	112/74	−16	4.6	4.8		20	16				
C. H.	154/108	−10	1.4	1.3		94	91				

* Indicates that tetraethylammonium chloride was given at time of maximum effect from Protoveratrine. In all cases 200 to 300 mg. of tetraethylammonium chloride were given i.v. so that ganglionic blockade was judged incomplete. Mean BP declines were minimal with tetraethylammonium chloride, never exceeding −10 per cent. Mean BP represents systolic plus diastolic÷2. Average values quoted represent the mean of 5 consecutive observations at 30 sec. intervals before and at the time of maximum drug action.

The numbers in italics represent mean values the differences of which are statistically significant with a probability of 1:20 or greater by analysis of variance.

cumbent patients observed after a half hour of quiet rest in a room maintained at an average temperature of 70 to 74° F. (Table III). With three exceptions there was a consistent increase in blood flow to the foot averaging 55 per cent and a lesser increase of 36 per cent in the forearm circulation, with a marked decrease in vascular resistance in both areas. The increases in blood flow were more marked following tetraethylammonium in a moderate dosage, and when the TEA was given at the height of Protoveratrine vasodilatation, further increases occurred (E. S. and M. A., Table III). Three patients were selected whose lower extremities had been denervated by lumbar sympathectomy so effectively that postoperative testing with TEA revealed no increase in blood flow. After the administration of comparable doses of Protoveratrine to these individuals, no increase in blood flow to the foot was observed and local vascular resistance either increased or was unchanged.

DISCUSSION

These observations indicate that moderate reductions in blood pressure after parenterally administered Protoveratrine are associated with a decline in cardiac output, while more marked reductions in blood pressure involve, in addition, decreases in total peripheral resistance. This is in agreement with other observations that cardiac output decreases after intravenously administered Protoveratrine (8). In a somewhat similar study of veratrum viride derivatives (9) no change in cardiac output occurred in the absence of congestive failure. However, these studies were performed at later intervals after administration of the drug and perhaps correspond more closely to our observation in the patient followed for three hours after the gradual reduction of blood pressure by the oral route, in which case a reduction in output was also not observed.

The mechanism whereby the reduction in cardiac output occurs cannot be established in the studies here reported. It is not likely that the drug has a depressant effect on the ventricular musculature, since at least in the heart-lung preparation (10) and in the various studies in animals it has not been shown to exert a negative inotropic effect. The reductions in peripheral and possibly in central venous pressure caused by veratrum alkaloids are probably of insufficient magnitude to be the sole cause of the lowered output and the fact that stroke volume is not appreciably reduced is further evidence against a direct cardiac action or a decrease in venous return. If increase in pulse rate alone can elevate the cardiac output as would appear to be the case from McMichael's studies on atropine (11), it might be postulated that marked decreases following Protoveratrine might depress the output in similar fashion.

Changes in vascular resistance following Protoveratrine were measured in the renal and peripheral circuits. In the kidneys, a decrease in renal resistance, due largely to the fall in blood pressure, occurred. Similar observations have been recorded by other investigators employing either Veriloid (9) or Protoveratrine (12). What vasodilatation there was may have been a passive response to the reduced blood pressure since the renal vessels are known to have considerable autonomous capacity to dilate as blood pressure falls. A moderate decline in mannitol clearance was also observed (9), but could not be confirmed when inulin was used to measure the glomerular filtration rate. This discrepancy was attributed to reabsorption of mannitol during oliguria induced by veratrum.

Blood flow is increased and vascular resistance is decreased in the forearm and foot. The vasodilatation, at least so far as the foot is concerned, depends on an intact sympathetic innervation since it can no longer be demonstrated in the sympathectomized extremity. This observation is in accordance with that of the authors previously cited (9).

In conclusion, it should be emphasized that the observations in acute studies may not necessarily apply to the effects of chronic administration of veratrum alkaloids. It is possible that under the prolonged effects of hypotension compensatory vasodilatation occurs, and the circulatory system reaches a new equilibrium with the restoration of cardiac output to normal. Thus, prolonged reduction in blood pressure following oral administration in one case was not accompanied by the decreases in output seen in acute experiments. Similarly, the inhibition of urine flow seen after acute administration is said to decrease after prolonged administration (13). Finally, despite a slight decrease in glomerular filtration rate in

some acute experiments, no progression of azotemia was observed in the chronic treatment of nephritic patients with this drug (3).

CONCLUSIONS

1. Protoveratrine, administered intravenously, produced a moderate decline in cardiac output, a definite decline in cardiac work, and sometimes a decrease in total peripheral resistance.

2. Renal blood flow was unchanged but glomerular filtration rate fell slightly and urine flow markedly in acute experiments.

3. Blood flow in the foot and forearm was increased moderately when the sympathetic nerves to the extremity were intact.

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STUDIES ON PHENYLKETONURIA. I. RESTRICTED PHENYLALANINE INTAKE IN PHENYLKETONURIA [1]

By MARVIN D. ARMSTRONG and FRANK H. TYLER

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biochemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

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The relationship between the biochemical defect in the hereditary condition, phenylketonuria (phenylpyruvic oligophrenia), and the accompanying mental defect is at the present time obscure. The work of Fölling (1) and Jervis (2, 3) has shown that the inheritance of the condition appears to be controlled by a single autosomal recessive gene. A series of investigations by Jervis and his collaborators (4–11) has demonstrated that the biochemical defect lies in a failure of the affected individuals to oxidize phenylalanine to tyrosine in the normal fashion. The major abnormal metabolites known to occur in this condition can arise as a logical consequence of the accumulation of large amounts of phenylalanine and the channelling of this phenylalanine into abnormal pathways. These metabolites are phenylalanine itself (8), phenylpyruvic acid (8), phenyllactic acid (8), and phenylacetylglutamine (12). Abnormally high levels of phenylalanine are found in serum (20 to 60 mg. per 100 ml.) and cerebrospinal fluid (6 to 8 mg. per 100 ml.) (9) and phenylpyruvic acid has been demonstrated in the serum of affected individuals (10). Recently it has been found that lesser amounts of other abnormal metabolites are excreted. These include *o*-hydroxyphenylacetic acid (13, 14) and products derived from tyrosine (13, 15) and tryptophan (15, 16). The manner in which these substances arise will be considered in later communications.

According to present concepts of the mechanism of inheritance, single genes are thought to influence single metabolic reactions. Phenylketonuria appears to be the result of a defect in a single gene (3), and the enzyme system concerned appears to be the liver system affecting the oxidation of phenylalanine (11). The mental defect, therefore, should follow as a result of this primary biochemical lesion. Several hypotheses might be proposed to explain the mental disorder. These might be grouped into two major categories; one proposing that some substance necessary for normal mental development or function is absent in phenylketonuria, and one that some substance with a toxic effect on the nervous system is produced. It has been suggested that phenylalanine itself, in high levels, might act as a toxic agent (17). Phenylacetic acid, which might be formed from phenylpyruvic acid, has been reported to be toxic to the central nervous system when present in large amounts (18); when phenylacetylglutamine was demonstrated to be excreted in considerable amounts in phenylketonuria, it was suggested that phenylacetic acid might be the agent responsible for the mental defect (12).

The plan of these experiments was to restrict the phenylalanine intake of phenylketonuric patients so that their blood phenylalanine level decreased to an essentially normal value and phenylpyruvic acid excretion decreased to a low amount or vanished. The phenylalanine intake then would be adjusted so that low serum levels would be maintained, and the patients would be observed for a period of several weeks in order to determine whether an objective change in mental ability occurred. It had already been reported that when a low-protein or protein-free diet was fed to a phenylketonuric patient, an immediate lowering of the phenylpyruvic acid excretion occurred, but that phenylpyruvic acid again began to be excreted in considerable amounts as breakdown of tissue protein continued (19). It was apparent, then, that it would be necessary to devise a phenylalanine-low or phenylalanine-free diet. Because most food proteins contain a remarkably uniform amount of phenylalanine (about 4 to 6 per cent) it was necessary to use either a phenylalanine-free

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protein hydrolysate or a synthetic diet in which the protein component was provided by a mixture of pure amino acids. The preparation of a phenylalanineless hydrolysate was feasible, since activated charcoal adsorbs phenylalanine quantitatively from an hydrolysate, but for technical reasons it was preferable to use an amino acid mixture in our initial experiments.

Children were reckoned to be the best potential subjects. In case the mental involvement were progressive and irreversible, a better chance of success in obtaining improved mentality might be expected, and, if any improvement occurred over as short a time as three months, known pathways of child development should make any progress more easily detected in a very young child than in an older individual at a different stage of development.

So far, five children with phenylketonuria have been studied with this restricted regimen. Somewhat equivocal results were obtained with the first two cases, both aged four and one-half years. Therefore, publication of the initial results has been withheld for two years until more definitive information could be obtained. A 13-month old phenylketonuric girl has now been carried for nine months on a restricted diet and has been followed subsequently for 15 months on a normal diet, and two seven-month old infants have been maintained three months. The results from all of these experiments have been strongly indicative that some substance deleterious to the nervous system is present in phenylketonuria and is responsible for some of its pathological manifestations.

After our initial experiments were completed, a report appeared of a similar experiment in which a two and one-half-year old child with phenylketonuria had been given a phenylalanineless casein hydrolysate (20), and recently a personal communication from another group described another such case (21). The results obtained by these workers, although divergent, are essentially in accord with the findings reported here and will be discussed later.

MATERIALS AND METHODS

The amino acids used in the synthetic diets were obtained from commercial sources and were used without further purification.

Fasting serum levels of phenylalanine were determined by a modification of the method of Kapeller-Adler (22). A trichloroacetic acid (TCA) filtrate of serum was extracted with ether to remove TCA, the aqueous solution was evaporated to dryness on a steam bath, color was developed as described by Block and Bolling (23) and was measured at 540 mμ with a Coleman Junior spectrophotometer. Satisfactory recoveries of phenylalanine added to serum were obtained under these conditions. Histidine and tyrosine did not interfere when the color was measured at 540 mμ but tryptophan gave rise to an absorbency about the same as that given by phenylalanine. The values for phenylalanine thus actually represent the sum of phenylalanine and tryptophan; error in the estimation of phenylalanine becomes significant only at low levels. The values obtained from a large number of measurements made on fasting normal controls were 3 to 5 mg. per 100 ml., which is in agreement with the reported average values of 2.0 mg. per 100 ml. phenylalanine and 1.7 mg. per 100 ml. tryptophan (combined, 3.7 mg. per 100 ml.) for fasting human sera (24).

Because of the difficulty of making extended 24-hour urine collections from mentally impaired children, analytical determinations were performed on spot collections of urine. Creatinine was determined by the Jaffé method, total N by Kjeldahl distillation, and keto acids by a modification of the method of Penrose and Quastel (19). Keto acid excretion is expressed as milligrams of phenylpyruvic acid per milligram of creatinine. A large number of determinations on different samples from the same individual and on samples from different individuals indicated that this provides a satisfactory measure of the phenylpyruvic acid excretion. The values relating keto acids to total urinary N and to creatinine were in agreement, so only the values relating the excretion to creatinine are reported here. On Figures 1–5, representative values for keto acid excretion are given at intervals, except during periods when significant changes occurred from day to day, when they are listed daily.

Electroencephalographic measurements were made as frequently as possible with a Grass 8 channel electroencephalograph.

SYNTHETIC DIETS

These experiments represent the first extended clinical use of synthetic diets, in which the protein component has been supplied in the form of a mixture of pure amino acids. Because of the possibility that other investigators might wish to use similar diets in other types of studies, and because several improvements have been made in compounding and administering these diets during the past 30 months, a discussion is warranted of the development of the diets currently in use.

The composition of the present amino acid mixture (Table I) differs in several noteworthy respects from the ones first prepared. The first diet used was patterned after those used for animal experiments in this

TABLE I

Phenylalanine-deficient amino acid mixture

	gm.
DL-Valine	30
DL-Isoleucine	30
L-Leucine	22
DL-Methionine	5
L-Lysine·HCl	20
DL-Threonine	14
L-Histidine·HCl·H_2O	10
L-Arginine·HCl	10
L-Cystine	3
L-Pyroglutamic acid	20
L-Asparagine·H_2O	20
DL-Alanine	20
Glycine	20
L-Tryptophan	0.4
L-Tyrosine	4
(Sodium bicarbonate)	10
	238.4

laboratory (25), and contained all of the commonly occurring amino acids with the exception of phenylalanine, serine, proline, and hydroxyproline. Serine was omitted because of the toxic effects that have been observed when *DL*-serine is given to young rats, and because of the prohibitive expense of *L*-serine; in addition, much recent work has shown that serine may be formed quite rapidly in the presence of an adequate supply of glycine and one-carbon compounds and may also be formed from carbohydrate in the presence of an adequate supply of nitrogen. Proline and hydroxyproline likewise are expensive and are made readily by animals. So far, no deleterious effect of a dietary deficiency of these amino acids has been demonstrated by animal experiments.

Some difficulty was encountered in the early days of the experiments because the patients balked at taking the diet and apparently suffered some nausea. The nausea which occurs after the ingestion of glutamic and aspartic acids in protein hydrolysates and amino acid mixtures has been known for some time. Despite this, it was not considered advisable to omit glutamic acid and aspartic acid from the basal mixtures, because of the central position occupied by glutamic acid in amino acid, carbohydrate, and fat metabolism and its known importance in the metabolism of nerve tissue. Accordingly, the possibility was considered of providing the acidic amino acids in the form of simple derivatives which might not have the bad side-effects of the free amino acids. *L*-Asparagine was adopted as an economical substitute for aspartic acid, but glutamine is too expensive to be considered as a substitute for glutamic acid. The readily prepared *L*-pyrollidonecarboxylic acid (pyroglutamic acid) was tested as a substitute for glutamic acid and proved to be quite satisfactory. In our experiments there was no indication of a change in nitrogen excretion when it was substituted for glutamic acid, and previous work with animals had already indicated it is absorbed and metabolized to a major extent (26). The incorporation of *L*-asparagine and *L*-pyroglutamic acid in lieu of aspartic acid and glutamic acid eliminated almost completely any noxious taste or nauseating after effects of the amino acid diet, and little trouble has been encountered in administering diets to patients after this change was made.

It should be noted that the amino acid mixture now in use contains a very low amount of *L*-tryptophan, which approximates 0.04 per cent in the total diet, and a small amount of *L*-tyrosine, roughly 0.4 per cent in the complete diet. In the first dietary experiment with Case 1, to be described later, 0.8 per cent of *DL*-tryptophan and 1.0 per cent of *L*-tyrosine were used. In the course of analytical work on urine from this patient, abnormal color reactions were noted; they were found to arise as a result of the use of this amount of *DL*-tryptophan. This observation subsequently led to the identification of abnormal indole acids as constituents of the urine of all patients with phenylketonuria (16). Rather than alter conditions in the middle of the first experiment with Case 1, however, her basal diet was left unchanged throughout its entire course. Case 2, started about four weeks later than Case 1, was given a diet containing 0.1 per cent of *L*-tryptophan, but the same higher level of tyrosine, while Case 3 was given a diet containing 0.04 per cent *L*-tryptophan. After Cases 1 and 2 had been returned to a natural diet and Case 3 had received the low-tryptophan phenylalanine-deficient diet for three months, the alteration to the use of pyroglutamic acid and asparagine in place of glutamic and aspartic acids was made. Further work, carried out toward the end of the experiment with Case 3, demonstrated the presence of elevated amounts of *p*-hydroxyphenyl acids in phenylketonuric urine, so the amount of tyrosine was reduced. Cases 4 and 5 are currently under study and are receiving the amino acid mixture described in Table I.

Water-soluble vitamins were prepared as a mixture in Celluflour® (Chicago Dietetic Association); this mixture was prepared in bulk (Table II) and stored in the cold. For convenience, a basal dry mix (Table III) sufficient to provide diet for several days was prepared and

TABLE II

Vitamin-Celluflour® mixture

	gm.
Riboflavin	2.50
Nicotinic acid	10.00
Pyridoxine·HCl	5.00
Calcium *d*-pantothenate	5.00
Thiamine·HCl	2.50
Folic acid	0.50
2-Methylnaphthoquinone	0.50
p-Aminobenzoic acid	10.00
Biotin	0.050
Vitamin B_{12}	0.025
Choline chloride	150.00
Ascorbic acid	40.00
Vitamins	226.075
Celluflour®	1000.00
Total	1226.075

TABLE III

Basal dry mix	gm.	Basal diet	gm./kg./day
Amino acids	228	Basal dry mix	4.2
Salt mixture	44	(Protein, estimated 3.0)	
Vitamins—		Carbohydrate	10.0
Celluflour®	12	Fat	3.0
	284		

stored in the cold. This mixture contained the amino acids, mineral salts (General Biochemicals, Inc., Salt Mixture No. 2), water soluble vitamins, and the small amount of Celluflour® included with the vitamin mixture. No apparent deterioration has been detected when this dry mixture has been allowed to stand at room temperature for as long as a year. Fat soluble vitamins were given daily in the form of the usual pharmaceutical preparations.

Diets were compounded in at least five or six portions daily from the basal dry mix, carbohydrate, and fat when they were provided as a slurry to suckling infants. When they were given to older children, the daily diet was prepared as a uniform paste which was stored in the cold and fed in portions. Because of the aim of maintaining low blood levels of phenylalanine, as many feedings as practical were spread throughout 24 hours of a day. Satisfactory quantities as determined in these experiments are given in Table III; individual variations in the demand for amino acids and total calories caused the need for minor adjustments on each patient. Fat was provided as Wesson oil or olive oil to infants suckling a bottle and as a hydrogenated vegetable oil (Spry, Crisco), to older children who ate a paste. Carbohydrate was provided as sucrose or dextrin.

Initial gastrointestinal distress invariably has been encountered for the first few days of the feeding of the synthetic diet. This is manifested as a watery diarrhea accompanied by inflammation and soreness of the perineal region, gas pains, and intestinal cramping. These phenomena usually abated after three to four days. Roughage was provided as Celluflour® to meet individual requirements. Usually, about 2 gm. per kg. per day is sufficient to allow normal bowel function for children.

Occasionally, children receiving the diets balked at consuming them and showed signs of distress after two to three days. This appeared to occur as a result of an excessive caloric intake and the problem usually could be overcome by reducing the amount of carbohydrate or fat in the diet in order to decrease the caloric intake, which then could be increased gradually to establish an optimal level. It was considered essential to retain a constant intake of amino acids, particularly during the depletion period, in order that breakdown of tissue protein would not lead to an accumulation of excess phenylalanine.

The basal phenylalanine-deficient diet was adhered to rigorously for the initial depletion periods during which the blood phenylalanine levels were lowered. With the first two cases studied, the diet was kept free of natural foodstuffs during most of the experimental period, with the exception of very small amounts of corn starch cookies, prepared as suggested by Rose, Johnson, and Haines (27); these were given frequently between meals along with strained butter and small amounts of clear jelly. Later, small amounts of low protein foods such as strained citrus fruit juices, bananas, apple sauce, cherry syrup, carbonated beverages, *etc.*, were used for flavoring in an attempt to alleviate the bad taste of the amino acid mixture, which at that time contained glutamic acid.

The approximate maximal level of phenylalanine intake above which serum levels began to increase was established by progressively increasing the daily intake of these children. This proved to be about 15 to 20 mg. per kg. per day. For the last three cases studied, an intake of 15 mg. per kg. per day has been used and has proved adequate in that this amount allowed the patients to gain weight while still maintaining a low blood level of phenylalanine. With these cases the 15 mg. phenylalanine per kg. per day was provided in the form of protein from natural foods. With infants, an appropriate amount of milk (assuming milk to contain approximately 4 per cent protein and milk protein to contain roughly 5 per cent phenylalanine) was used. With older children, small amounts of low-protein baby foods, such as strained applesauce, pears, and peaches and some vegetable preparations were used; in this case the analyses of the foods furnished by the manufacturer were used to approximate the amount of protein in the food and the proteins were assumed to contain 5 per cent phenylalanine. The use of natural foodstuffs in this manner possesses some advantages: first, it has a sparing action on the other amino acids which necessarily accompany the phenylalanine and thus cuts down to some extent the use of the expensive amino acids; second, it provides a more esthetic medium (for the administerer if not the patient) with which to flavor the diets, and finally, it provides more natural roughage than is obtained by the use of Celluflour® alone.

EXPERIMENTAL

When these experiments were undertaken, there was some question as to whether restricting the phenylalanine intake of patients with phenylketonuria would result in a lowering of their levels of blood and tissue phenylalanine to a normal range, or whether they might keep somewhat elevated blood levels, go into negative nitrogen balance, and lose weight. The results obtained with all five cases have been similar: there was no marked loss of weight associated with the administration of restricted diets, blood levels of phenylalanine rapidly came down to a normal range, and the excretion of phenylpyruvic acid ceased within three days after the diet was commenced. The

data concerning serum phenylalanine levels, urinary excretion of phenylpyruvic acid, phenylalanine intake and weights are presented graphically in Figures 1 to 5, which are included with the appropriate case histories. The excretion of the abnormal metabolites, most of which had not been characterized adequately at the time the first three cases were studied, was followed with the last two cases. It is significant to note here that the excretion of these metabolites decreased to approximately normal amounts as blood levels of phenylalanine approached a normal value; the nature, mechanism of formation, and possible significance of these metabolites will be discussed in detail in later publications.

Case 1

This patient was a four and one-half-year old girl (Born 11/13/47). She had lived at home until she was admitted to the hospital in May, 1952. Retardation of development was not noted by the parents until she was eight months of age, at which time she was still unable to sit without support. At one year she began to sit up and could use a few words. She learned to walk at two years but did not develop further in language ability and gradually lost the few words she had acquired. Her gait never became normal but was stiff-legged and hypermetric. She had been hyperactive and noisy, frequently banging her head on the wall, slapping her face, voluntarily falling down, biting her tongue, and screaming unintelligibly. There had been no episodes of unconsciousness or convulsions.

Family history: The patient has three sisters. The two eldest are normal. The third child also is a phenylketonuric; she is severely retarded and is confined in a state institution.

Physical examination: T—100° (R); P—104; R—24; BP—106/72; Wt.—17.3 kg.; Ht.—100 cm. This husky red-headed female child had an extensive, red, scaling dermatitis of the forearms and legs and the characteristic odor of phenylacetic acid. She was extremely hyperactive, her attention passing from one point to another in rapid progression. Her face was round and ruddy in appearance, and her hair was light red in color. She grated her teeth together nearly continually and frequently uttered unrecognizable cries but no words. She was able to walk well, although her movements tended to be hypermetric but not dysmetric. She was able to pick things up and could climb over nearly any obstacle. She responded to attention with grimacing but did not play with toys or feed herself.

General physical examination revealed no positive findings. The skin was fair and soft to the touch except in the areas of dermatitis. The subcutaneous tissue was softer than normal but not otherwise remarkable. Neurologic examination showed that her muscular tone was somewhat increased generally and that reflexes were hyperactive and equal. Plantar response was flexor. No tremor was present. No objective evidence of sensory impairment was apparent.

Laboratory data: The volume of packed red cells was 38 per cent. The remainder of the usual blood and urine examinations were normal. The Kahn was negative. The acidified urine developed a deep green color on the addition of ferric chloride; the fasting serum phenylalanine level was 42 mg. per 100 ml.

Chest and skull were normal on X-ray examination. A technically unsatisfactory electroencephalogram showed some slow waves but no seizure discharges; no further electroencephalographic measurements were made.

Hospital course: After an uneventful control period of 10 days during which she gained to 18 kg. on a normal diet, she was placed on an artificial diet in which her protein need was met with the phenylalanine deficient diet. She accepted this poorly and had some watery diarrhea but maintained her weight, until she developed a respiratory infection with low grade fever during the third week. Her weight fell to 16.5 kg. Subsequently, she did well physically.

She refused the diet and remained in a semi-fasting condition from the 8th to the 13th day of the deficient diet. During this period, as would be expected, tissue protein breakdown continued and on Figure 1 it should be noted that sufficient phenylalanine accumulated to raise the serum level from 6 mg. per 100 ml. to 10 mg. per 100 ml. Beginning with the fifteenth day her food intake was enforced, and by the 23rd day of the diet she had a serum phenylalanine level of 4 mg. per 100 ml., an essentially normal level with the analytical method used.

Rather than risk exceeding the amount of phenylalanine she could tolerate, the daily allowance was increased gradually throughout the experimental period. It is evident that only when the amount was increased to 500 mg. per day (30 mg. per kg. per day) was there a gradual increase in her blood phenylalanine level; in 10 days it had increased from 4 mg. per 100 ml. to 7 mg. per 100 ml. In spite of this rise, phenylpyruvic acid excretion did not recur.

During the first four weeks in the hospital she adapted to the ward situation and showed some improvements in behavior which appeared to be related to the dietary regimen. These consisted of a moderate decrease in the hyperactivity and a considerable increase in her attention span on direct stimulation by an examiner, so that she could be entertained for a period of several minutes. She would also engage in types of continued activity of her own choosing for periods as long as 15 to 20 minutes. The dermatitis disappeared after the first week of the diet and her skin remained clear for the remainder of her hospital stay.

For the last eight weeks she appeared to regress, gradually became more hyperactive, her attention span decreased, and at the end of 12 weeks her behavior was very similar to that at the onset of the regimen. When

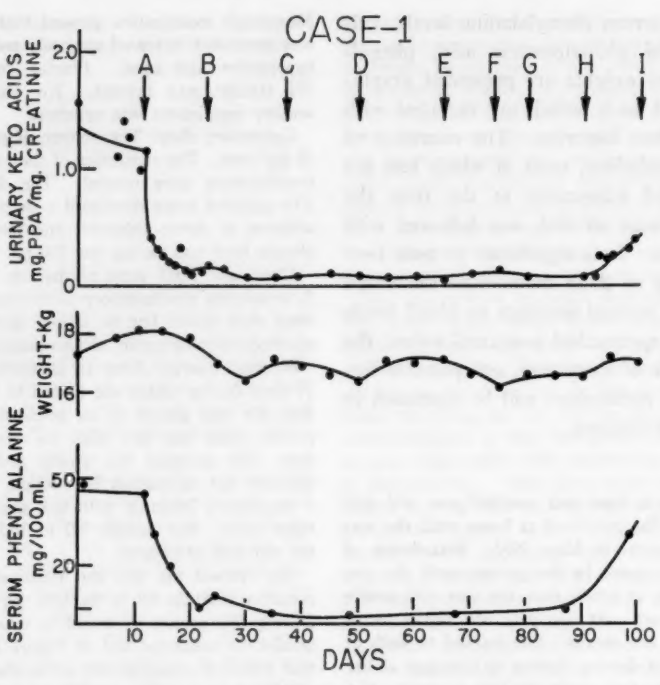

FIGURE 1

A. Phenylalanine-deficient diet initiated
B. 90 mg. *L*-phenylalanine daily
C. 100 mg. *L*-phenylalanine daily
D. 110 mg. *L*-phenylalanine daily
E. 120 mg. *L*-phenylalanine daily
F. 250 mg. *L*-phenylalanine daily
G. 500 mg. *L*-phenylalanine daily
H. 1000 mg. *L*-phenylalanine daily
I. Natural diet

she was returned to a natural diet no striking change occurred and she was discharged home.

Course at home: On a natural diet at home her dermatitis recurred. She has continued to grow normally during the following 22 months. She is now a very stout six-year old, who uses a few recognizable words and continues with her marked hyperactivity; her attention span is still limited to a few minutes. She appears to have progressed at about the same relative rate as for the preceding four years.

Case 2

This patient was a three-year and eleven-month old boy (Born 7/8/48) with extreme mental retardation. He was admitted from the Utah State Training School in June, 1952. He was the product of a normal pregnancy and was thought to be normal during the neonatal period and up to the age of five months. At this time a severe otitis media developed, and subsequent to this period he became obviously retarded. Recurrent "eczema" was a problem at this time. An umbilical hernia was successfully repaired at six months. He failed to learn to sit up. At seven months he began to have seizures which sometimes extended to clonic movements of the arms and legs. These became progressively more severe and were not controlled by anticonvulsive medication. He became more retarded and was practically unresponsive, although he continued to take food and grew satisfactorily. He was admitted to the state institution where he was maintained on heavy phenobarbital sedation (0.3 gm. per day). Although he remained physically well except for an attack of scarlet fever two months before admission, he had led a vegetative existence and never learned to sit up or play. He responded very little to the attendants and continued to have numerous convulsive seizures throughout this period.

Family history: This patient has two siblings with phenylketonuria (Cases 3 and 5) and a normal eight-year old sister. The parents are living and well. Three collaterals of the maternal great-grandmother are known to have been mentally retarded or to have had seizures. Three paternal great-aunts had convulsive disorders and died in infancy.

Physical examination: T—99.4; P—80; R—20; BP—105/50; Wt.—16 kg. This thin, blond, pale-skinned boy was unresponsive to examination or toys. Intermittently

he developed opisthotonos with irregular clonic movements of the arms and blinking of the eyes. Between episodes he chewed on his arms. The head appeared slightly large for his thin body but the fontanelles were closed and the maximum circumference of the head was only 50 cm. Ear drums showed old scars but no evidence of recent infection. His teeth were small, cone shaped and serrated. The chest was thin with prominent sternum and flaring costal margins. The abdomen showed only a liver palpable 1 cm. below the costal margin. On neurological examination there was increased muscular tone with "cog-wheel" rigidity of arms and legs between periods of seizures. Tendon reflexes were hyperactive but equal. Plantar responses were flexor. No abnormal reflexes were demonstrated. Sensation could not be tested. When frightened, he exhibited an abrupt stiffening with tremors of the extremities, and his skin showed large blotchy red areas. He did not have a dermatitis at this time.

Laboratory data: The usual clinical examination of the blood, urine, and blood chemistry showed no abnormalities. Blood Kahn was negative. Addition of ferric chloride to the acidified urine produced a deep greenish-black color. The fasting serum phenylalanine level was 42 mg. per 100 ml. Skull roentgenograms showed a relatively small cranial vault with a sloping anterior table. Electroencephalography showed abnormal activity with bursts of diffuse slow waves and periods of petit mal variant activity, most prominently displayed over the occipital lobes.

Hospital course: During the initial two-week period on the ward, the phenobarbital was discontinued. Although he continued to have many convulsive seizures they were less frequent than formerly. He was somewhat more responsive than he had been previously but continued to be unable to sit up and paid little attention to toys. The experience gained with feeding the first case made possible a more satisfactory initial and continued feeding of the experimental diet to this patient. He gradually gained weight from a minimum of 14 kg. early in the experimental period to a weight of 17 kg. toward

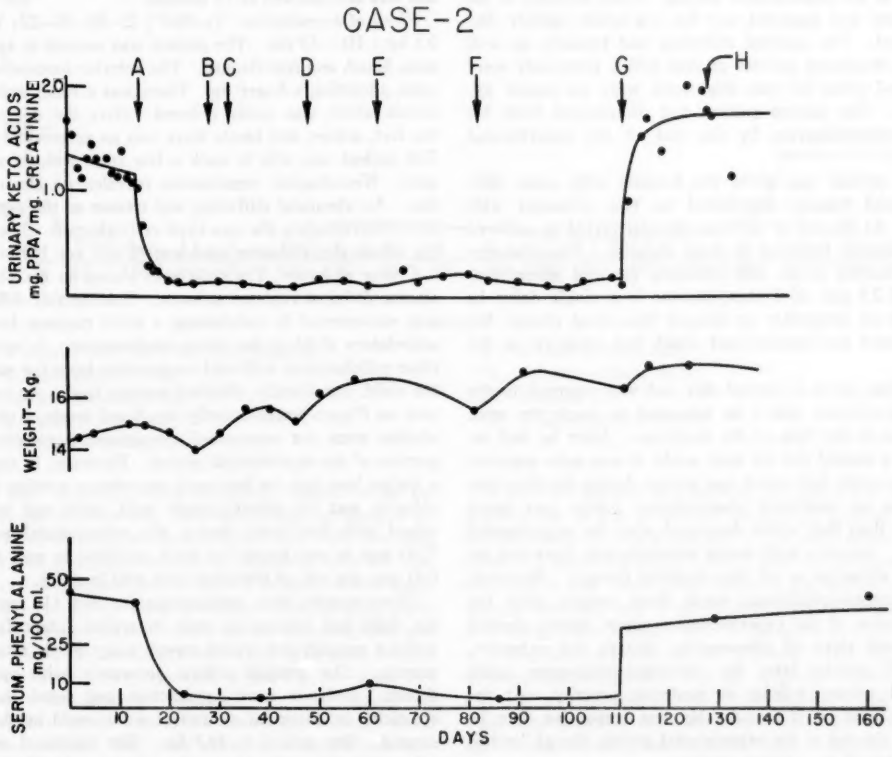

FIGURE 2

A. Phenylalanine-deficient diet initiated
B. 200 mg. *L*-phenylalanine daily
C. 250 mg. *L*-phenylalanine daily
D. 300 mg. *L*-phenylalanine daily
E. 200 mg. *L*-phenylalanine daily
F. 250 mg. *L*-phenylalanine daily
G. 2.5 gm. *L*-phenylalanine daily
H. Natural diet

the close; this gain represents an improvement, while receiving the restricted diet, of a malnourished condition which was the consequence of an attack of scarlet fever shortly before he was brought into the metabolic ward. Little experimentation was carried out to determine critically the maximum phenylalanine intake which he could tolerate without increasing his blood phenylalanine levels. It was, however, established that 300 mg. per day (19 mg. per kg. per day) was sufficient to cause an increase in blood phenylalanine and that 200 mg. (12.5 mg. per kg. per day) allowed a lowering of the increased level. It seems probable that 15 to 17 mg. per kg. per day of phenylalanine would have provided an adequate amount of phenylalanine but would not have furnished an excess which could accumulate.

After the experimental diet had been instituted, his seizures disappeared completely and over a period of several weeks he learned to sit up, eventually pulling himself up to the sitting position. He became quite responsive to examiners and nurses and played with toys for brief periods. He remained in this state for the last six weeks of the experimental period. Some decrease in his spasticity was apparent and the cog-wheel rigidity disappeared. The marked stiffening and tremors, as well as the discolored patches of skin which previously were observed when he was frightened were no longer apparent. The seizure pattern had disappeared from his electroencephalogram by the end of the experimental period.

The patient was given the formula with some difficulty and became dehydrated on two occasions with fever. At the end of the experimental period he suffered a respiratory infection of short duration. Phenylalanine was returned to his diet following the oral administration of 2.5 gm. of *L*-phenylalanine in a single dose; he showed no immediate or delayed behavioral change but maintained the improvement which had occurred on the diet.

He was given a natural diet and was returned to the state institution, where he remained in much the same state as at the time of his discharge. After he had received a normal diet for four weeks it was quite apparent that the scalp hair which had grown during the time that he was on restricted phenylalanine intake was much darker than that which developed after the experimental period. Seizures with motor manifestations have not recurred either on or off phenobarbital therapy. However, an electroencephalogram made three months after the termination of the experimental regimen clearly showed occasional signs of abnormality, though not extensive, and 20 months later his electroencephalogram again showed seizure activity of moderate severity. At the present time he is considerably less responsive than he was at the end of the experimental period, though he still pulls himself to a sitting position.

Case 3

This infant girl is a sibling of Cases 2 and 5 and was first seen at the age of 13 months (Born 7/3/51). At that time her mother noted that she had the same odor which had been pointed out to her as occurring with the older child. Pregnancy and delivery were uneventful, except that the patient was born one month prematurely, and weighed only 2500 gm. She seemed to be entirely normal during the first months of life. At five months she developed a skin rash which was thought by her pediatrician to be the result of a food allergy. In spite of dietary changes this recurred at intervals. A small umbilical hernia noted shortly after birth had gradually lessened.

Developmentally, she had seemed entirely normal to the parents and pediatrician during the first year. She followed movements with her eyes at one month, sat erect at six months and stood alone and pulled herself erect at 10 months. At one year of age she walked with the support of a chair and said two or three words intelligible to the parents. Consultation with the pediatrician who had followed the child revealed a normal development, with the exception of marked tremulousness of voluntary activity which was prominent at the age of 11 months and was still marked at 13 months.

Physical examination: T—98.6°; P—96; R—22; Wt.—9.1 kg.; Ht.—53 cm. The patient was normal in appearance, blond, and fair-skinned. The anterior fontanelle was open, admitting a finger tip. There was a small umbilical hernia which was easily reduced. Over the dorsum of the feet, ankles, and hands there was an eczematoid rash. The patient was able to walk a few steps without assistance. Neurological examination revealed no abnormalities. An abnormal stiffening and tremor of the extremities occurred when she was tired or frightened. The fasting serum phenylalanine level was 42 mg. per 100 ml.

Course at home: The child was placed on the phenylalanine deficient regimen at home. Considerable difficulty was encountered in maintaining a strict regimen for this ambulatory child in her home environment. In spite of close collaboration with and cooperation from the parents the child occasionally obtained protein food. As can be seen on Figure 3 satisfactorily low blood levels of phenylalanine were not maintained throughout a considerable portion of the experimental period. However, in spite of a higher base line for keto-acid excretion a positive ferric chloride test for phenylpyruvic acid could not be obtained with her urine during the experimental period. This test in our hands has been sensitive to well under 0.01 mg. per ml. of phenylpyruvic acid in urine.

Three months after commencing the diet (16 months) the child had learned to walk in typical infant fashion without support and would repeat many words after the parents. She grasped objects accurately with opposed thumb. Reflexes were hyperactive and equal and no spasticity or abnormal pyramidal signs could be demonstrated. She gained to 10.3 kg. She continued on the controlled diet until 22 months of age. At 20 months she had chicken pox with moderate associated rhinopharyngitis and excoriation and superficial infection of the skin lesions. Her respiratory infection continued and at 22 months she began to vomit the diet and lost weight

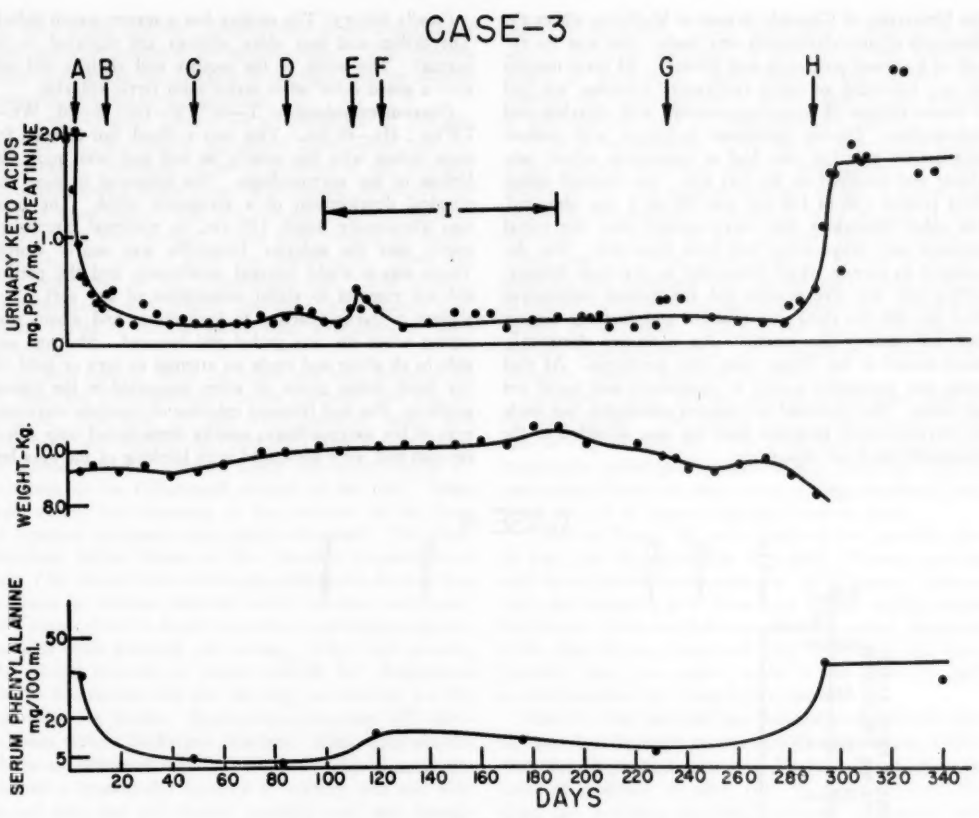

FIGURE 3

A. Phenylalanine-deficient diet initiated
B. 1.5 gm. protein daily
C. 2.5 gm. protein daily
D. 3.0 gm. protein daily
E. Accidental normal meal
F. 2.0 gm. protein daily
G. 3.0 gm. protein daily
H. Natural diet
I. Child given jelly beans

to 8.6 kg. Although these difficulties seemed to be related to the infection and were improving, the parents insisted on returning her to a normal diet. At this time she showed a motor development consistent with her age. She used a number (about 12) of simple words spontaneously but did not form sentences. She identified parts of the body on request. Her mother reported that she played with a doll which she fed · t the table with a spoon from her plate. She was able to put on her shoes but laced them inaccurately. Her gait was "stiff legged" but no spasticity or other abnormal neurologic findings could be demonstrated. One week after she returned to a natural diet she was examined by a psychologist, who used several tests. Her average performance, as well as could be judged, at this time corresponded to that of an 18-month old child (chronological age 23 months, I.Q. 78).

When she returned to a natural diet there was no immediate loss of the motor and intellectual abilities which she had attained. She was tested by the same psychologist under the same conditions after a year had elapsed. At this time it was evident that she had not maintained the slightly retarded rate of development she showed a year previously. She was assessed as performing at the level of a 23-month old child (C.A. 35 months, I.Q. 65). She is particularly retarded now in language development. Physical examination reveals slight spasticity and somewhat hypermetric movements suggestive of extra-pyramidal disease, but no other abnormal long tract signs.

Case 4

This baby girl was first observed in May, 1954 at the age of nine months (Born 8/9/53). She had been followed since November, 1953, by Dr. Robert H. Alway of

the University of Colorado School of Medicine, where the diagnosis of phenylketonuria was made. She was the result of a normal pregnancy and delivery. At three months of age, following an upper respiratory infection, she had a severe episode of bronchopneumonia with diarrhea and dehydration. During parenteral hydration with sodium bicarbonate solution, she had a convulsion which was clonic and localized on the left side. An elevated spinal fluid protein (96 to 136 mg. per 100 ml.) was observed, but other laboratory data were normal after the initial acidosis and dehydration had been corrected. She developed an anemia which responded to oral iron therapy. When she was five months old the mother commented that she felt the child was retarded and that the diapers had an "unholy-like smell." An extensive dermatitis, most severe in the diaper area, had developed. At that time, she responded poorly to examiners and could not sit alone. She continued to progress physically, but made no developmental progress until she was admitted to the metabolic ward for this study.

Family history: The mother has a severe speech defect. The father and two older siblings are reported to be normal. The urine of the mother and siblings did not give a green color when tested with ferric chloride.

Physical examination: T—98°; P—110; R—30; Wt.—7.2 kg.; Ht.—61 cm. This was a blond, fair-skinned female infant who lay quietly in bed and was quite oblivious of her surroundings. She appeared to have the physical development of a six-month child. The head was abnormally small (39 cm. in maximal circumference), and the anterior fontanelle was nearly closed. There was a slight internal strabismus, and the patient did not respond to visual stimulation of any sort. She showed a startle reaction to loud noises and would stop crying when she was patted and bounced. She was unable to sit alone and made no attempt to turn or hold up her head, either prone or when supported in the sitting position. She had frequent episodes of complete unawareness of her surroundings; usually these lasted only a few seconds and were associated with blinking of the eyes but

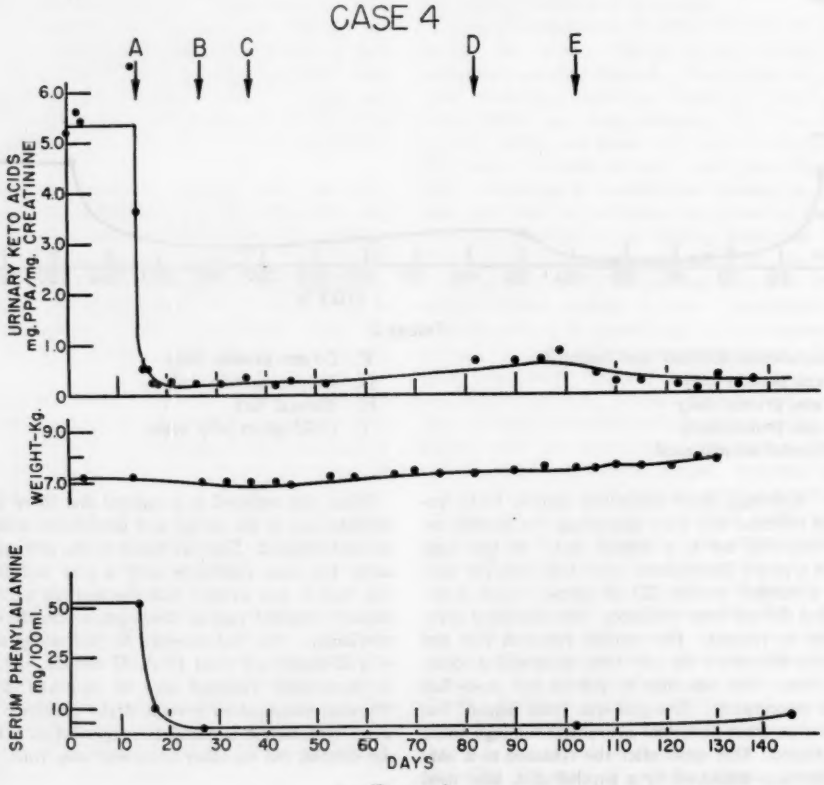

FIGURE 4

A. Phenylalanine-deficient diet initiated
B. Two gm. protein daily
C. Four gm. protein daily
D. Commence phenylalanine-deficient modified casein hydrolysate in place of amino acids
E. Five gm. protein daily

no other motor activity. Examination of the lungs, heart and abdomen revealed no abnormalities. The reflexes were hyperactive, particularly in the legs, and the plantar response was extensor bilaterally.

Laboratory data: Except for a hemoglobin of 11.0 gm. per 100 ml., the routine laboratory examination was not remarkable. Addition of ferric chloride to the acidified urine produced a green color. Her fasting serum phenylalanine level was 53 mg. per 100 ml. Roentgenograms of the chest were normal. Skull films showed no abnormalities; the sutures were not remarkable. Bone age was equivalent to the chronologic age. The electroencephalogram was grossly abnormal with almost continual bursts of diffuse paroxysmal discharges of high amplitude.

Hospital course: After the infant had been observed for 12 days, the phenylalanine-deficient synthetic diet was instituted. She took the diet well, appeared happy and contented, and suffered no discomfort with the exception of a mild recurrent diarrhea which was controlled by alterations in the Celluflour® content of the diet. After two weeks, the frequency of the seizures in the form of lapses of awareness was clearly decreased. The characteristic diffuse bursts of the infantile hypsarhythmic type (28) found in the electroencephalogram became less frequent in records obtained under standard conditions. She appeared to be more responsive to attention, and responded with giggling and cooing. After two months, the clinical evidence of seizure activity had disappeared almost completely and she has had no seizures for the following six weeks. Electroencephalograms still demonstrate seizure discharges, however. After three months of the experimental diet (at twelve months of age), she showed a considerable increase in activity, and was able to roll over and pull herself partially erect and demonstrated improved posture of her head. Her strabismus had disappeared and she responded to visual stimuli, but she did not play with toys or grasp accurately. The restricted regimen is being continued. At the present time, she is still grossly retarded, but appears to have made more progress during the three months on phenylalanine restriction than during the preceding eight months. As nearly as can be judged, she appears to have gained from an approximate mental age of three months to an age of six months during the experimental period.

Case 5

This boy is a sibling of Cases 2 and 3 (Born 9/2/53). The pregnancy and neonatal course were uneventful. The parents reported he developed normally until April 24, 1954 (seven and one-half months); he had been seen casually by us at intervals throughout his early infancy and had appeared to be developing normally. He had a slight scaling dermatitis. His hair was a dark red color for the first two months, and lightened rapidly to a light blond color during the next three months. The third week in April he appeared to be drowsy and in distress. He was irritable and clutched at his head and left ear. He had his first seizure on April 24; over a period of a week an increasing number of seizures with some motor manifestations occurred. They were then replaced by an almost continual sequence of seizures in the form of lapses of awareness. According to the parents, the schedule of demonstration of these seizures was similar to that observed with his older brother (Case 2).

Physical examination: This was a pale, blond eight-month old boy of normal physical development. He was unable to sit. He showed frequent lapses of awareness of one to two minutes in duration, with periods of consciousness of three to five minutes between seizures. There were no motor manifestations. During these episodes his eyes remained open, but did not respond to light, movement of objects, nor did they close when approached closely by an object. His reflexes were very hyperactive and were symmetrical, with patellar and ankle clonus elicitable; plantar response was extensor.

Laboratory data: Routine clinical examination showed no abnormality. The fasting serum phenylalanine level was 63 mg. per 100 ml. The electroencephalogram was maximally pathological, with closely recurrent diffuse paroxysmal bursts of slow waves of large amplitude, and short periods of damped activity between bursts.

Course at home: He was placed on the synthetic diet at home and has taken this very well. Physical growth and development have continued in a normal pattern, with the exception of a three-week period, during which he became threonine-deficient. This occurred inadvertently when he was transferred from a synthetic diet compounded from pure amino acids to one prepared with a phenylalanine-free casein hydrolysate.

After he had received the phenylalanine-deficient diet for two days he began to have grand mal seizures, rather than the lapses of awareness. He had six or seven of these of moderate duration (two to three minutes) the third day, and they gradually decreased in number each day and in the duration of each attack, until the 22nd day, on which he had no detectable seizure. He continued to have an occasional seizure of 15 to 30 seconds duration until the 33rd day, and had none thereafter. Electroencephalograms were taken frequently during this period, and showed steady improvement until the 33rd day. At this time the electroencephalogram was normal for an infant of his age. His appearance had changed during this time from one of indolence and disinterest to that of a normal alert infant.

After the patient had been seizure-free and with a normal electroencephalogram for 11 days, phenylalanine was returned to the synthetic diet. There was no immediate sign of deterioration, but in the course of the next two weeks he showed increasing signs of distress and irritability. He also demonstrated evidence of neurological impairment. These included pooling of secretions in the pharynx, increased briskness of the tendon reflexes, and manifest patellar clonus. He showed an increasing number of periods of lapses of awareness, resembling those observed when he was first seen. These were different, however, in that they were of shorter duration and he could be aroused from them by sudden noises; he could not be aroused from the similar type of

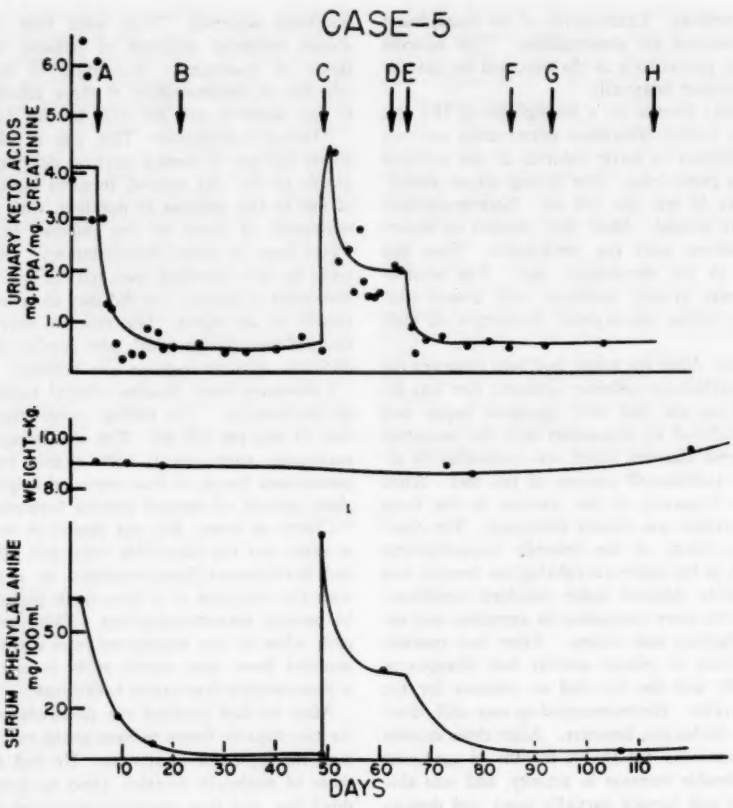

FIGURE 5

A. Phenylalanine-deficient diet initiated
B. 2.4 gm. protein daily
C. 6.0 gm. *L*-phenylalanine over 90 minutes, then 850 mg. *L*-phenylalanine daily
D. 1300 mg. *L*-phenylalanine daily
E. Phenylalanine-deficient modified casein hydrolysate
F. 2.4 gm. protein daily
G. 3.6 gm. protein daily
H. 4.8 gm. protein daily

seizure occurring at the beginning of the experimental period. An unexpected finding was that his electroencephalogram remained normal throughout this period!

In order not to risk the possibility of permanent damage to this infant, he was returned to the deficient regimen after he had received phenylalanine for 16 days.[2] At the present time (12 months of age) he is able to stand with support but does not pull himself erect. He grasps with fair accuracy, using an opposed thumb, and examines objects briefly before discarding them or placing them in his mouth. He makes various sounds but has no recognizable words. Overall, his developmental age appears to be about two months behind his chronological age, and he seems to have progressed at nearly a normal rate following the delay occasioned by his illness at seven months of age. It is planned to continue providing him with the diet for as long as possible.

[2] At this time he was given a basal diet made up from a phenylalanine-free casein hydrolysate, which was also devoid of tyrosine, tryptophan, glutamic acid and aspartic acid. The hydrolysate was supplemented with amino acids to make it approximate in composition the basal amino acid mixture listed in Table I.

DISCUSSION

Restriction of the phenylalanine intake of these patients with phenylketonuria led to a lowering of their blood levels of phenylalanine to normal values. Their excretion of phenylpyruvic acid ceased when the serum phenylalanine fell below 15 to 17 mg. per 100 ml. and the amounts of the minor

abnormal metabolites, *o*-hydroxyphenylacetic acid, *p*-hydroxyphenyllactic acid, *p*-hydroxyphenylacetic acid, indolelactic acid, and indoleacetic acid which were excreted, decreased to approximately normal as the serum phenylalanine reached a normal level. When phenylalanine was returned to their diets the high blood level of phenylalanine was restored immediately, and the abnormal metabolites again began to be excreted. Thus, the prominent biochemical abnormalities which characterize the condition were corrected by this regimen.

The changes in the mental and neurological status of the patients were not so definite, but several conclusions concerning the nature of the mental defect occurring in phenylketonuria may be drawn as a result of the observations presented here. First, it is apparent that the oligophrenia in phenylketonuria does not occur simply as a result of a functional interference in an otherwise normal brain either by the presence of excessive amounts of phenylalanine or by any of the abnormal metabolites which are formed as a consequence of its presence. None of the four patients who have been given a restricted regimen followed by a return to normal phenylalanine intake and the resulting high blood levels of this amino acid showed any immediate signs of a marked deterioration when phenylalanine was returned to their diet, in spite of the prompt recurrence of the biochemical anomalies. A marked and immediate regression certainly should have been expected if the mental symptoms were the result of inhibition of normal mental functioning in an otherwise normal central nervous system. In addition, Case 1 had demonstrated little change in behavior at the end of three months of the restricted regimen.

Improvement in some clinical aspects was observed, however, in each of the five cases studied, while they were receiving the synthetic diet. It seems certain that at least some of the pathological manifestations of phenylketonuria result because of the presence in affected individuals of some substance or substances with a detrimental effect upon the nervous system. This was particularly evidenced by the control of convulsions when they were present. With Case 5, a severe convulsive disorder was controlled within three weeks after the beginning of a phenylalanine-low diet. Also, with Case 2 seizures were eliminated and seizure discharges disappeared from the electroencephalogram during the three months of the restricted diet. Overt signs of epileptic seizures were eliminated in Case 4, although she still has a very abnormal electroencephalogram after three months. It seems likely that the clinical improvement observed in these three cases may have followed the correction of the grossly abnormal electrical activity of their brains. Other less objective signs which indicated a removal of some toxic agent in the patients studied included the disappearance or improvement of signs of neurological abnormality, elimination of the characteristic dermatitis found in phenylketonuria, an increased response to environmental stimuli, and a prolonged attention span. It should be emphasized here that no improvement would be expected if the pathological symptoms occur because of the *lack* of some essential substance; this regimen could not have provided a missing substance, but could have decreased the amount of an abnormal toxic substance. The slower rate of progress of Case 3 after she was returned to a natural diet is probably significant in indicating that she may have been protected from deterioration while she was receiving the restricted diet.

It seems probable that unknown and possibly complex factors other than the known defect in phenylalanine oxidation operate in conjunction with it and contribute to the mental defect. This is consistent with the findings of previous workers, who did not observe any difference in phenylalanine metabolism that could be related to the degree of mental defect, despite wide variations in the degree of mental impairment (9). The biochemical observations did not reveal any significant differences among any of the five patients studied in this work, yet there were marked differences in their clinical condition. This was particularly marked in the case of the two infants. Case 5 progressed in an apparently normal fashion until he was seven months old, while Case 4 was markedly retarded from a very early age, perhaps from birth. Case 3, who appeared at 13 months to be normal in physical and mental development, showed the same typical pattern of high blood phenylalanine and high urinary phenylpyruvic acid excretion as the other patients, including her brothers, Cases 2 and 5. Studies on the other abnormal metabolites excreted by these patients have failed to demonstrate any significant difference

between severely affected and slightly affected patients.

After the experiments were completed with the first three patients reported here, a description of a similar experiment was made by Bickel, Gerrard, and Hickmans (20). These investigators fed a diet containing a phenylalanineless casein hydrolysate to a severely affected two-year old phenylketonuric child. They reported a significant improvement in her condition over a period of several months. She took an interest in her surroundings, and developed much improved motor abilities. When 4 to 5 gm. of phenylalanine was added to the diet each day a prompt and striking regression to her earlier status occurred. The authors note, however, that, as in the case with our experimental subjects, it was difficult to make a critical assessment of the child's mental progress.

Recently, a communication from another British group (21) also reported a dietary experiment with a phenylketonuric child. Their two-year old patient was given a well-defined diet in which the nitrogen was provided in the form of pure amino acids, and the blood phenylalanine was maintained at a normal level (1.5 mg. per 100 ml.) for six months. No unambiguous changes in the patient's condition, objectively or subjectively, were observed, nor was there a change when phenylalanine was returned to the diet. The apparent discrepancy between the results of the British groups as well as the differences observed in our phenylketonuric patients may, however, only point to the complexity of the problem of relating the mental defect to the inherited defect in the metabolism of phenylalanine.

Whether the administration of diets restricted in phenylalanine will provide an effective therapeutic measure for phenylketonuria will await much further experimentation before any positive conclusions can be made. The results of our experiments may be interpreted as indicating that such diets are probably beneficial, particularly when a convulsive disorder is present. At the present time, however, it appears that such a regimen must be initiated at a very early age and very soon after the onset of pathological symptoms, to prevent permanent damage to the central nervous system. It might be speculated that the different experiences reported here with Cases 4 and 5, both of whom were started on a restricted diet at about the same age and who had the same outward signs of clinical involvement at the time they were first observed, occurred because Case 4 had been severely affected for at least six months, while Case 5 was given a restricted diet within three weeks after the onset of pathological symptoms. Permanent damage may have occurred to Case 4 during the longer period during which she suffered severe retardation of development.

That benefit can be derived from restricting the phenylalanine intake of phenylketonuric infants is well shown by Case 5, who is at present doing well. A control of sorts on this infant is provided by his older sibling, Case 2. The children bear a strong familial resemblance to each other, and both showed a nearly normal development until the age of six or seven months. At this time, both suffered the onset of a convulsive disorder, which continued unabated in Case 2 for several years, and which was completely controlled in Case 5 after he had received a phenylalanine-deficient diet for only three weeks.

Another point that remains to be settled is whether continuation of the dietary regimen will benefit patients who do not show an immediate apparent response. If the mental defect occurs primarily because of a lack of development of the brain rather than as the result of permanent damage, it is reasonable to conclude that the relatively short term experiments reported with four of our cases may not have been sufficiently long for the manifestation of a developing mentality. Further long term and well-controlled experiments with older children will need to be carried out to gather information in this connection.

SUMMARY

1. Well defined synthetic diets, in which the protein portion is composed of pure amino acids, have been prepared for use in clinical experiments.

2. Five children with phenylketonuria have been given synthetic diets deficient in phenylalanine. Blood phenylalanine levels were lowered to normal values, phenylpyruvic acid excretion ceased, and abnormally high excretion of other metabolites ceased. The children were maintained in this condition for varying lengths of time.

3. A four and one-half-year old girl demonstrated some behavioral improvement after four

weeks of this treatment and regressed to her original condition after three months.

4. A four and one-half-year old boy showed considerable improvement in behavior and in neurological status. The occurrence of frequent seizures ceased and seizure activity disappeared from his electroencephalogram. He did not revert to his previous condition rapidly when phenylalanine was returned to his diet, but deteriorated slowly.

5. A 13-month old girl, who was nearly normal in development, continued to develop at the same rate for nine months while receiving a phenylalanine-deficient diet. She has matured mentally more slowly during the 15 months following the return of phenylalanine to her diet.

6. A 10-month old girl was relieved of seizures, although her electroencephalogram has not become normal. She has developed much more rapidly while receiving the diet for three months than she had previously.

7. Almost continuous epileptiform seizures in an eight-month old boy were controlled completely and his electroencephalogram became normal after he had received the restricted diet for three weeks. When phenylalanine was returned to his diet for two weeks he did not show a recurrence of the seizures, but demonstrated signs of distress and of neurological impairment. At present he is continuing to develop normally on the diet.

8. All the observations made on the patients with phenylketonuria studied are consistent with the hypothesis that the effects of some detrimental substance on the central nervous system have been overcome by the use of the phenylalanine-restricted diet. It seems probable that such diets should be initiated at a very early age in order to prevent irreversible damage to the central nervous system.

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We are especially indebted to Dr. R. H. Alway, Professor of Pediatrics, University of Colorado School of Medicine, who observed and made the diagnosis of phenylketonuria for Case 4 at the age of five months, and arranged to make her available for our current study and for a long-term continuation of the dietary regimen with her.

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EFFECTS OF HEPARIN ON THE LIPOPROTEINS IN HYPERLIPEMIA. AN ELECTROPHORETIC STUDY OF THE SERUM ALPHA AND BETA LIPOPROTEINS AFTER THEIR SEPARATION BY FRACTIONATION OF THE PLASMA PROTEINS OR ULTRACENTRIFUGAL FLOTATION [1]

By FRANZ S. M. HERBST, WALTER F. LEVER, MARY E. LYONS, AND NANCY A. HURLEY

(From the Department of Dermatology, Harvard Medical School, and the Dermatologic Research Laboratory, Massachusetts General Hospital, Boston, Mass.)

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The effects of heparin on the electrophoretic pattern of serum have recently been studied in this Laboratory. It had been found that in normal, strictly fasting individuals an intravenous injection of heparin caused no changes in the patterns obtained either by moving boundary electrophoresis or by paper electrophoresis (1–4). On the other hand, in alimentary hyperlipemia and idiopathic hyperlipemia electrophoretic changes were observed. In the patterns obtained by moving boundary electrophoresis the changes consisted of a decrease in the beta-1 globulins to a subnormal value and the appearance of a new component ahead of albumin (1, 2). On the basis of this observation it had been assumed that the pre-albumin component consisted of beta lipoproteins. However, subsequent studies by paper electrophoresis revealed that after an injection of heparin not only the beta lipoproteins but also the alpha lipoproteins showed an acceleration of their electrophoretic migration: The beta lipoprotein lipid band moved with the speed of either alpha-2 globulin, alpha-1 globulin, or intermediary between alpha-1 globulin and albumin and the alpha lipoprotein lipid band moved faster than albumin (3, 4).

Because the findings by paper electrophoresis indicated that the pre-albumin component consisted of the alpha lipoproteins and not of the beta-lipoproteins, as we had originally assumed, it was thought worth while to study in greater detail the effects of heparin on the alpha and beta lipoproteins after their separation from one another. Consequently, electrophoretic analyses were carried out on plasma protein fractions and on the lipoproteins isolated by ultracentrifugal flotation in 10 normal fasting persons, two persons with alimentary hyperlipemia and seven patients with idiopathic hyperlipemia before as well as one hour after an intravenous injection of 100 mg. of heparin.

METHODS

Collection of blood. Blood was withdrawn in a dry syringe, the samples were allowed to clot at room temperature, then centrifuged and the serum removed with a pipette. For the fractionation of the plasma proteins another blood sample was withdrawn in a syringe containing acid citrate-dextrose solution as an anticoagulant (5).

Electrophoresis. Paper electrophoresis was carried out, as described in our previous communications (3, 4), according to the technique of Grassmann and Hannig (6) with subsequent staining of the proteins with Amido Black 10B, and of the lipids with Sudan Black B (7, 8). For the quantitative determination of the relative amounts of protein the paper strips stained for protein were immersed for half an hour in a mixture of alpha bromnaphthalene and paraffin oil. This produced sufficient clearing of the paper strips so that they could be read in a densitometer. The diagram thus obtained was analyzed by planimetry. Quantitative determination of the relative amounts of lipids on the lipid stained paper strips gave variable and unreliable results which therefore are not reported.

Moving boundary electrophoresis was performed in a Perkin-Elmer Tiselius electrophoresis apparatus, Model 38, as described in previous communications (3, 9).

Fractionation. Fractionation of the plasma proteins was carried out according to Method 10 of Cohn and his co-workers (10), modified for small amounts of plasma by Lever and his co-workers (5). Two main fractions were obtained, Fraction I + II + III, containing the beta lipoproteins, and Fraction IV + V, containing the alpha lipoproteins. Since Fraction I + II + III contained

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fibrinogen, which on analysis by paper electrophoresis interacts with the supporting medium, two drops of a thrombin solution containing 200 N.I.H. units per ml. were added to the fraction, following which the fibrin clot was removed. Electrophoretic analysis was carried out on the two main fractions within twenty-four hours after the fractionation of the plasma.

Preparative ultracentrifugation. Spinco Model L was used as preparative ultracentrifuge. The rotor was Spinco Model No. 40 in which the tubes are fixed at an angle of 26 degrees. Centrifugation was carried out at 16 C. for approximately fifteen hours at a speed of 35,000 R.P.M. which is equivalent to a gravitational force of 80,730 G.

For preparative ultracentrifugation at a solution density of 1.063 Gm. per ml. 3 ml. of serum were mixed with 7 ml. of a sodium chloride-water solution of such density to give a final density of 1.063 Gm. per ml. at 26 C. After centrifugation the uppermost 1 ml. containing the low density lipoproteins, or beta lipoproteins, was then pipetted off (11).

For preparative ultracentrifugation at a solution density of 1.21 Gm. per ml. 3 ml. of serum were mixed with 7 ml. of a potassium bromide-water solution of such density to give a final density of 1.21 Gm. per ml. at 26 C. After centrifugation the uppermost 1 ml. was pipetted off. In it were present the low as well as the high density lipoproteins, *i.e.,* the alpha and beta lipoproteins (12, 13).

Paper electrophoresis of the isolated lipoproteins was carried out within twenty-four hours after ultracentrifugation.

RESULTS

Plasma fractions

Fasting normal persons. Fraction IV + V on analysis by paper electrophoresis and subsequent staining for proteins contained, just as on analysis by moving boundary electrophoresis (10, 5), most of the albumin of the serum, most of the alpha-1 globulins, and small amounts of the alpha-2 and beta globulins (Figure 1). On the paper strips stained for lipids one lipid band was present in the alpha-1 globulin area representing the alpha lipoproteins.

Fraction I + II + III showed on the paper strips stained for proteins, just as on moving boundary electrophoresis, small amounts of albumin, most of the alpha-2 and beta globulins and all the gamma globulin (Figure 2). Paper strips stained for lipids showed one lipid band in the beta-1 globulin area representing the beta lipoproteins.

Injection of heparin into 10 normal fasting persons caused no changes in the paper electrophoretic patterns of either Fraction IV + V or Fraction I + II + III.

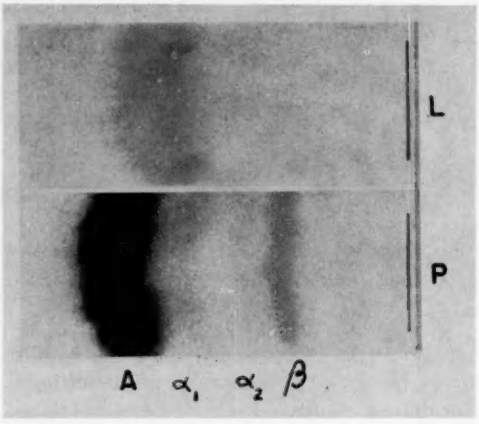

FIG. 1. PAPER ELECTROPHORETIC PATTERNS OF THE ALPHA LIPOPROTEIN FRACTION (FRACTION IV + V) OF A NORMAL INDIVIDUAL

On the lipid stained paper strip (L) one band is present in the alpha-1 globulin area, representing the alpha lipoproteins.

Idiopathic hyperlipemia. Analysis of Fraction IV + V (alpha lipoprotein fraction) by paper electrophoresis and subsequent staining for proteins and lipids in seven patients with idiopathic hyperlipemia revealed a normal pattern. One

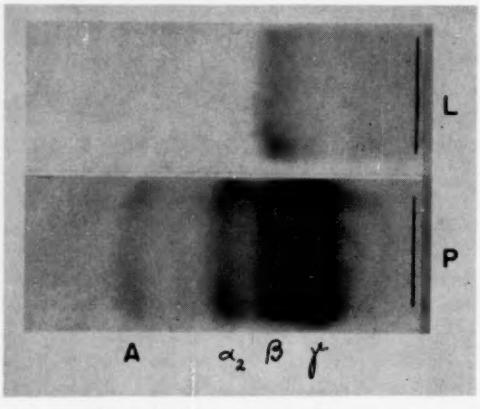

FIG. 2. PAPER ELECTROPHORETIC PATTERNS OF THE BETA LIPOPROTEIN FRACTION (FRACTION I + II + III) OF A NORMAL INDIVIDUAL

On the lipid stained paper strip (L) one band is present in the beta globulin area, representing the beta lipoproteins.

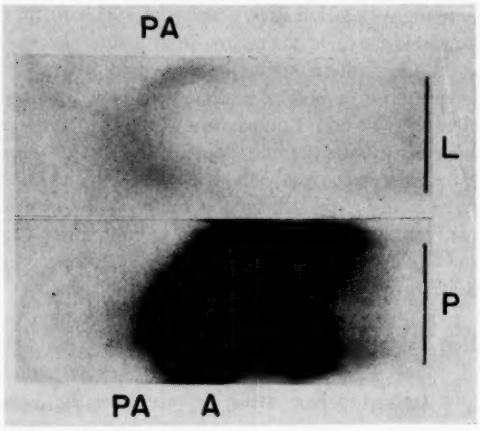

FIG. 3. PAPER ELECTROPHORETIC PATTERNS OF THE ALPHA LIPOPROTEIN FRACTION (FRACTION IV + V) OF A PATIENT WITH IDIOPATHIC HYPERLIPEMIA AFTER AN INTRAVENOUS INJECTION OF 100 MG. OF HEPARIN

The alpha lipoprotein band on the lipid stained paper strip (L) moves faster even than albumin as a pre-albumin component (PA). The protein stained paper strip (P) shows that the pre-albumin component contains a small amount of protein.

hour after the intravenous injection of 100 mg. of heparin, staining for lipid revealed that the one lipid band present (alpha lipoprotein) had increased its speed of migration and migrated ahead of albumin (Figure 3). The protein stained paper strip showed that this pre-albumin component contained small amounts of protein. Analysis of Fraction IV + V by moving boundary electrophoresis also showed the pre-albumin component (Figure 4).

Analysis of Fraction I + II + III (beta lipoprotein fraction) by paper electrophoresis and subsequent staining for proteins revealed a greater intensity of staining of the beta globulin band than in normal fasting persons. On staining for lipids the lipid band present in the beta globulin area (beta lipoprotein) stained much more intensely than normal. Furthermore, just as in the patterns obtained on whole serum (4), the beta lipoprotein band showed a densely stained trail extending back to the starting line. This trail was due mainly to the chylomicra. One hour after the injection of heparin, the trail of the beta lipoprotein band showed a considerable decrease in its staining intensity and the beta lipoprotein band itself moved with the speed of alpha-2 or even alpha-1 globulin (Figure 5). The protein stained paper strip showed a small amount of protein moving with the same increased speed as the beta lipoprotein on the lipid stained paper, repre-

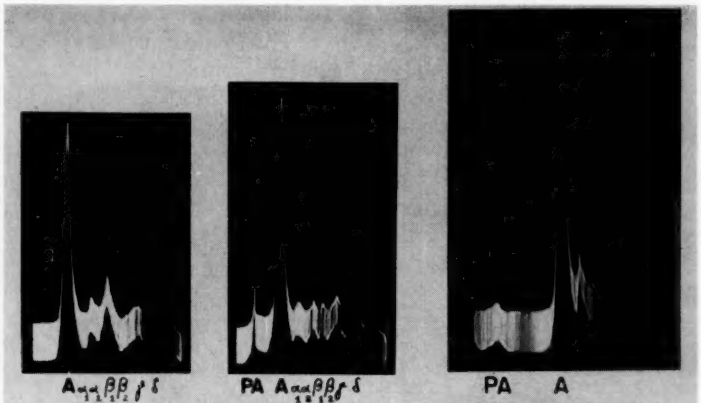

FIG. 4. PATTERNS OBTAINED BY MOVING BOUNDARY ELECTROPHORESIS IN A PATIENT WITH IDIOPATHIC HYPERLIPEMIA

Left, fasting serum: the alpha-2 peak is slightly and the beta-1 peak is moderately elevated. Middle, serum after an intravenous injection of 100 mg. of heparin: the beta-1 peak has decreased to a subnormal size and a new component has appeared ahead of albumin (PA). Right, alpha lipoprotein fraction (Fraction IV + V) after an intravenous injection of heparin: The pre-albumin component (PA) is present in this fraction.

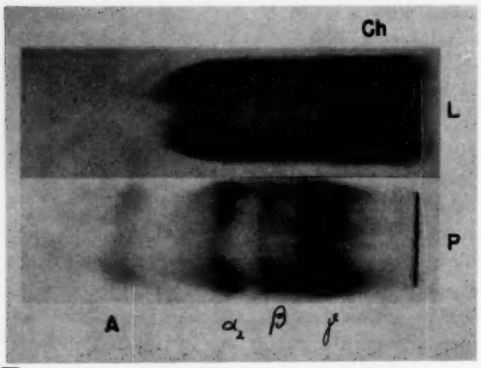

FIG. 5. PAPER ELECTROPHORETIC PATTERNS OF THE BETA LIPOPROTEIN FRACTION (FRACTION I + II + III) OF A PATIENT WITH IDIOPATHIC HYPERLIPEMIA AFTER AN INTRAVENOUS INJECTION OF 100 MG. OF HEPARIN

The beta lipoprotein band on the lipid stained paper strip (L) moves with the speed of alpha-1 globulin.

senting the protein moiety of the beta lipoproteins. Satisfactory patterns by the moving boundary method could not be obtained because of the milky appearance of Fraction I + II + III, caused by the presence of large amounts of chylomicra.

Isolated lipoproteins

Fasting normal persons. Paper electrophoresis carried out on the lipoproteins isolated by ultracentrifugal flotation at a density of 1.21 Gm. per ml. showed two bands on the lipid stained as well as on the protein stained paper strip indicating the presence of both the alpha and beta lipoproteins (Figure 6). Comparison of the lipid stained paper strip with the one stained for proteins confirmed the fact, established already by chemical analysis (14), that the alpha lipoproteins have a relatively low lipid-protein ratio in contrast to the beta lipoproteins which have a high lipid-protein ratio.

Paper electrophoresis of the lipoproteins isolated by flotation at a density of 1.063 Gm. per ml. showed only one band on the protein and lipid stained paper strips representing the beta lipoproteins (Figure 7). Again the relatively high lipid-protein ratio of the beta lipoproteins was apparent.

Injection of heparin into 10 fasting normal persons did not cause any changes in the electrophoretic patterns.

Idiopathic hyperlipemia. Analysis by paper electrophoresis of the lipoproteins floated at a density of 1.21 Gm. per ml. (alpha and beta lipoproteins) revealed two distinct bands on both the protein and lipid stained paper strips representing the alpha and beta lipoproteins, respectively. The intensity of staining of the beta lipoproteins was greater than normal on the protein stained

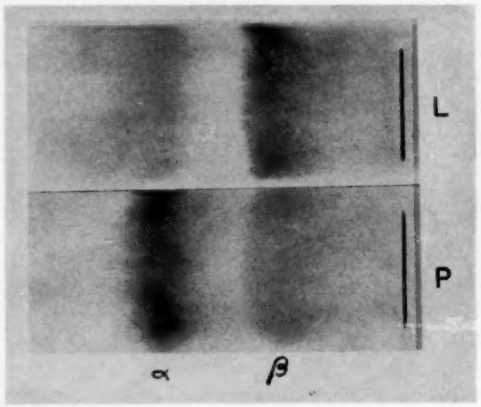

FIG. 6. PAPER ELECTROPHORETIC PATTERNS OF THE LIPOPROTEINS OF A NORMAL INDIVIDUAL ISOLATED BY ULTRACENTRIFUGAL FLOTATION AT A DENSITY OF 1.21 GM. PER ML.

Two bands representing the alpha and beta lipoproteins, respectively, are present on the lipid stained (L) as well as on the protein stained (P) paper strip.

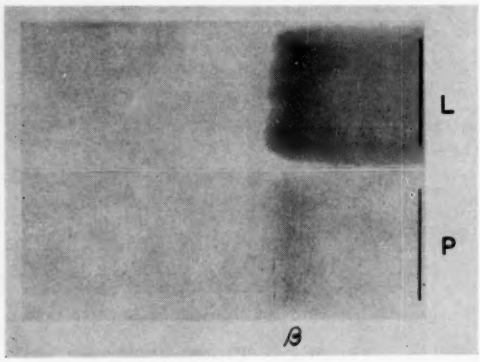

FIG. 7. PAPER ELECTROPHORETIC PATTERNS OF THE LIPOPROTEINS OF A NORMAL INDIVIDUAL ISOLATED BY ULTRACENTRIFUGAL FLOTATION AT A DENSITY OF 1.063 GM. PER ML.

One band representing the beta lipoproteins is present on the lipid stained (L) and on the protein stained (P) paper strips.

as well as on the lipid stained paper strip. As in the analysis of whole serum and of Fraction I + II + III, the lipid stained paper strip but not the protein stained paper strip showed considerable trailing of the beta lipoprotein band, due to the chylomicra.

Analysis of the lipoproteins floated at a density of 1.063 Gm. per ml. (beta lipoproteins) revealed the same patterns as just described except that no alpha lipoprotein band was present.

An injection of heparin caused the following changes in the lipoproteins floated at a density of 1.21 Gm .per ml. (alpha and beta lipoproteins): On the lipid stained paper strip the alpha lipoprotein band appeared much broader than before and the trail of the beta lipoprotein band representing the chylomicra showed a decrease in its staining intensity. On the protein stained paper strip three bands were now present instead of two bands (Figure 8). Comparison of the protein stained with the lipid stained paper strip indicated that only the slowest moving of the three bands on the protein stained paper strip corresponded to the beta lipoproteins, whereas the two faster moving bands corresponded to the alpha lipoproteins. That the two faster moving bands represented the alpha lipoproteins and only the third, slowest moving band represented the beta lipoproteins was definitely proven by analysis of the beta lipoproteins floated at a density of 1.063 Gm. per ml. since paper electrophoresis of the beta lipoproteins thus obtained showed only one band on the protein and lipid stained paper strips corresponding to the slowest moving band.

In order to obtain information to which extent heparin had caused an increase in the speed of electrophoretic migration of the lipoproteins, blood was obtained before and after the injection of heparin and the lipoproteins were isolated by flotation at a density of 1.21 Gm. per ml. They then were analyzed side by side in the same electrophoresis chamber and both paper strips were stained for proteins. Comparison of the pre-heparin sample with the post-heparin sample revealed after the administration of heparin an increase in the electrophoretic mobility of the alpha as well as of the beta lipoproteins (Figure 8). The alpha lipoprotein band had split up into two bands of which the slower band showed only a slight increase in mobility, was narrow and well stained, while the faster band was broad and faintly stained.

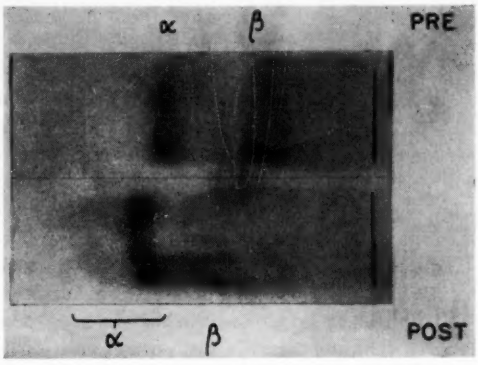

FIG. 8. PAPER ELECTROPHORETIC PATTERNS (STAINED FOR PROTEINS) OF THE LIPOPROTEINS OF A PATIENT WITH IDIOPATHIC HYPERLIPEMIA BEFORE (PRE) AND AFTER (POST) AN INTRAVENOUS INJECTION OF HEPARIN

The lipoproteins were isolated by ultracentrifugal flotation at a density of 1.21 Gm. per ml. The pre-heparin and post-heparin samples were analyzed simultaneously. The alpha lipoproteins in the post-heparin sample are split up into two components. Both alpha lipoprotein bands as well as the beta lipoprotein band show an increase in their electrophoretic mobility following the injection of heparin.

Alimentary hyperlipemia. Two normal persons in an alimentary hyperlipemic state were studied. They had received a meal consisting of 480 ml. of heavy cream, 32 Gm. of sugar and 16 Gm. of cocoa. Three and a half hours after the meal 100 mg. of heparin were injected intravenously. Analysis by paper electrophoresis of the plasma fractions and of the isolated lipoproteins before and after heparin gave the same results as in idiopathic hyperlipemia, except that the beta lipoproteins on the protein-stained as well as on the lipid stained paper strip showed a normal staining intensity.

Determination of the relative distribution of proteins between alpha and beta lipoproteins

The alpha and beta lipoproteins of the serum were isolated by flotation at a density of 1.21 Gm. per ml. before and after an injection of heparin. They then were analyzed by paper electrophoresis and the paper strips were stained for protein.

Determination of the relative amounts of pro-

tein present in the alpha and beta lipoprotein bands of the paper strips revealed in 10 fasting normal individuals, on the average, 74 per cent of the proteins as alpha lipoproteins and 26 per cent as beta lipoproteins (range of variation 24 to 27 per cent). The average values in seven patients with idiopathic hyperlipemia revealed a considerably higher percentage of proteins in the beta lipoprotein band than in normal persons, namely 42 instead of 26 per cent (range of variation 36 to 51 per cent). This was indicative of the great increase in beta lipoproteins in this disease. The relative values obtained in two normal individuals in an alimentary hyperlipemic state were the same as in normal fasting persons.

Injection of heparin into 10 normal persons, seven patients with idiopathic hyperlipemia and two normal persons in an alimentary hyperlipemic state caused no changes in the distribution of proteins between the alpha and beta lipoproteins in any of the cases.

DISCUSSION

For a study of the action of heparin on the lipoproteins of normal and hyperlipemic sera two methods were employed by us: Separation of the alpha lipoproteins from the beta lipoproteins by chemical fractionation, and isolation of the two lipoproteins by ultracentrifugal flotation at densities of 1.063 and 1.21 Gm. per ml. These two methods in conjunction with analysis by paper electrophoresis proved very useful since they enabled us to study the effects of heparin on the alpha and beta lipoproteins separately. The analyses by paper electrophoresis showed that fractionation of the plasma proteins by the small-scale modification of Cohn's Method 10 resulted in a clear-cut separation of the alpha lipoproteins from the beta lipoproteins since each was present in one fraction exclusively. Even after the injection of heparin complete separation of the two lipoproteins was obtained.

Effects of heparin on the electrophoretic mobility of the lipoproteins

Injection of heparin caused no change in the electrophoretic mobility of the separated or isolated lipoproteins in normal individuals. On the other hand, in persons with either alimentary or idiopathic hyperlipemia both the alpha and beta lipoproteins showed an increase in their electrophoretic mobility. Of especial interest were the findings on chemical fractionation where after the injection of heparin the beta lipoproteins on the paper strip used for the analysis of Fraction I + II + III moved with the speed of alpha-2 or alpha-1 globulin, while the alpha lipoproteins on the paper strip used for the analysis of Fraction IV + V moved faster than albumin. This provided conclusive evidence that the pre-albumin component which we had observed following the injection of heparin in the electrophoretic patterns of whole serum in idiopathic hyperlipemia (1, 2) as well as in alimentary hyperlipemia (3) represented the alpha lipoproteins.

Does heparin cause a transformation of beta into alpha lipoproteins?

Several authors (15–19) have expressed the opinion that heparin causes a transformation of beta lipoproteins into alpha lipoproteins. Boyle, Bragdon, and Brown (15), carrying out ultracentrifugal analyses at a density of 1.21 Gm. per ml., observed in alimentary hyperlipemia on injection of heparin and subsequent *in vitro* incubation of the plasma an increase in the S_f classes representing the alpha lipoproteins. Nikkilä (18), who also made *in vivo* studies, found by analysis with moving boundary and paper electrophoresis an increase in the amount of the alpha lipoprotein lipids in alimentary hyperlipemia following the injection of heparin. This observation, however, merely indicates that the beta lipoproteins migrated with the speed of the alpha lipoproteins and does not indicate an actual transformation of beta lipoproteins into alpha lipoproteins.[2]

[2] The fact that Nikkilä observed no pre-albumin component in alimentary hyperlipemia after the injection of heparin may be due to the fact that he gave smaller amounts of cream (200 ml.) than we did (480 ml.) for the purpose of inducing alimentary hyperlipemia and consequently only the beta lipoproteins but not the alpha lipoproteins showed an increase in the speed of electrophoretic migration. This would be analogous to an observation which we made on two individuals whom we had presumed to be fasting but who had not been strictly fasting (2). On moving boundary electrophoresis we observed in both persons a decrease in the beta-1 globulin peak from normal to subnormal values together with an increase in the alpha-2 peak in one and in the alpha-1 peak in the other. There was no pre-albumin peak present in either of these two persons.

The following are our reasons for believing that an *in vivo* transformation of beta lipoproteins into alpha lipoproteins by heparin does not occur: First, analysis of whole serum by paper electrophoresis and subsequent staining for lipids never showed an increase in the staining intensity of the alpha lipoprotein band after the injection of heparin either in alimentary hyperlipemia (3) or idiopathic hyperlipemia (4). Second, as shown in the present study, isolation of the two lipoproteins by ultracentrifugal flotation followed by paper electrophoresis and staining for proteins showed after the injection of heparin neither in alimentary nor in idiopathic hyperlipemia any change in the distribution of the proteins between alpha and beta lipoproteins. Third, chemical fractionation of the plasma proteins by Cohn's Method 10 and determination of the amounts of cholesterol and phospholipids present as alpha lipoproteins and beta lipoproteins in idiopathic hyperlipemia never revealed abnormally high amounts of lipids in the alpha lipoprotein fraction either after a single injection or after the long-term administration of heparin; whereas the elevated values for cholesterol and phospholipids in the beta lipoprotein fraction decreased (1, 20). Fourth, heparin was found to exert no effect on beta lipoproteins with S_f values below 12 (21). Although beta lipoproteins with high S_f values were successively transformed into beta lipoproteins with lower S_f values but slight changes only occurred in those normal individuals whose serum contained solely beta lipoproteins with S_f values of 20 and below; and no changes took place in normal individuals who had only beta lipoproteins with S_f values below 12. Since the gradual transformation of the low density beta lipoproteins into those of higher density stops after the beta lipoproteins have incurred a certain increase in density it seems unlikely that the beta lipoproteins could change all the way into high density alpha lipoproteins.

Cause of the increase in electrophoretic mobility of the alpha and beta lipoproteins after heparin

According to our present knowledge heparin has a direct effect only on beta lipoproteins of high S_f values causing their transformation into those of lower S_f values, but apparently has no effect on beta lipoproteins of low S_f values and on the alpha lipoproteins. How then does heparin cause an increase in the electrophoretic mobility of the alpha as well as of the beta lipoproteins in hyperlipemic sera? It appears likely that these electrophoretic changes are a secondary effect of the action of heparin on the beta lipoproteins with high S_f values. Shore, Nichols, and Freeman (22) observed that *in vivo* heparinized plasma caused a release of fatty acids from beta lipoproteins provided they were obtained from lipemic sera and thus possessed high S_f values. Furthermore, Gordon (23) by the *in vitro* addition of oleate to normal serum produced an increase in the mobility of the alpha and beta lipoproteins.

If one assumes that the increase in the electrophoretic mobility of the alpha and beta lipoproteins after an injection of heparin is due to the binding of fatty acids by these lipoproteins, as suggested by Gordon's study, one could explain not only the increased mobility of both lipoprotein classes in persons with hyperlipemia but also the absence of electrophoretic changes in strictly fasting normal persons. Fasting serum contains beta lipoproteins of low S_f values only. Thus very small amounts of fatty acids are available for release by heparin. On the other hand, hyperlipemic serum contains large amounts of beta lipoproteins with high S_f values and accordingly large amounts of fatty acids (24). Some of these fatty acids are released after an injection of heparin and then bound to the beta lipoproteins of low S_f values as well as to the alpha lipoproteins, thus increasing the electrophoretic mobility of these lipoproteins.

SUMMARY

1. The action of heparin on the serum alpha and beta lipoproteins in idiopathic and alimentary hyperlipemia was studied by electrophoretic analyses of these two lipoproteins after their separation by chemical fractionation as well as after their isolation by ultracentrifugal flotation at densities of 1.063 and 1.21 Gm. per ml.

2. Electrophoretic analysis of the alpha and beta lipoproteins after their chemical separation revealed in both types of hyperlipemia as the result of an intravenous injection of heparin an increase in the electrophoretic mobility of both lipoproteins. The separated alpha lipoproteins moved

faster than albumin and the separated beta lipoproteins moved with the speed of either alpha-2 or alpha-1 globulin.

3. Electrophoretic analysis of the alpha and beta lipoproteins after their isolation by ultracentrifugal flotation also revealed an increase in their electrophoretic mobility following the injection of heparin. The alpha lipoproteins were present as two components in the post-heparin patterns obtained by paper electrophoresis.

4. These studies allow the conclusion that the pre-albumin component which we had observed following the injection of heparin in the electrophoretic patterns of whole serum in idiopathic as well as alimentary hyperlipemia represents the alpha lipoproteins.

5. No evidence was found in this study as well as in our previous studies to support the recently proposed concept that the administration of heparin causes a transformation of beta lipoproteins into alpha lipoproteins.

6. The increase in the electrophoretic mobility of the alpha and beta lipoproteins in hyperlipemia following the administration of heparin may be explained by the lipolytic effect of heparin and the resultant binding of fatty acids by the alpha and beta lipoproteins.

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THE CALORIC VALUE OF LABILE BODY TISSUE IN OBESE SUBJECTS

By VINCENT P. DOLE, IRVING L. SCHWARTZ, NIELS A. THORN, AND LAWRENCE SILVER

(From the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.)

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The caloric balance normally varies from moment to moment. Food is taken intermittently, absorbed, stored, and used as needed for the continuous requirements of metabolism. Variations of diet and of physical activity on successive days cause temporary inequalities of energy intake and output. Long-term balance requires some system to smooth these fluctuations.

In the present work we have endeavored to define one aspect of the system by correlating small changes in body weight with known variations of caloric intake. The ratio of these two quantities —the caloric equivalent of labile body weight— sets an upper limit to the energy value of primary storage tissue.

The caloric equivalent would be easy to measure if all changes in weight were due to caloric imbalance. In reality, however, various other processes—menstrual changes or venous congestion, for instance—cause body fluid to vary without relation to the caloric status of the individual. Any single measurement of energy balance and weight change fails to define the caloric equivalent because it provides no means to distinguish a specific change of weight from an accidental fluctuation of water balance.

Measurement of the total fluid balance cannot resolve the difficulty, however, because a considerable amount of water is held intracellularly with glycogen and protein. If these substances are used for temporary storage of energy their accumulation and discharge will cause an obligate variation of fluid balance, quantitatively related to each caloric change, and thus part of the caloric equivalent. Possibly the volume of extracellular fluid also varies with the caloric response. In any case, the distinction between caloric and noncaloric changes of hydration can be made only by repetition of the measurement, since a specific response to caloric variation is defined solely by the fact of its consistent occurrence.

Standard methods for measurement of heat production—a calorimeter room (1) or an exact determination of water loss (2)—would be inconvenient for use in prolonged experiments such as are required by repeated changes of dietary intake. Fortunately, the average energy balance and the caloric equivalent can be measured more easily, and with greater accuracy, by an analysis of the weight response alone. Two known levels of dietary intake are equivalent in this case to the usual pair of data: one known level of intake and the measured rate of heat production. An additional advantage of the method is that it measures the caloric equivalent and the average daily requirement for maintenance under conditions that simulate normal variations of diet.

METHODS

Five obese but otherwise healthy women remained on the metabolic ward during the entire study. Each morning, after they had voided, a nurse weighed them on calibrated scales with a beam graduated in divisions of 100 gm. During the day they followed the usual routine of the ward: rest hour at 1 P. M., bedtime at 10 P. M.; walking, reading and light handiwork within the confines of ward and porch at other times. Their physical activity under these conditions appeared to remain nearly constant from day to day.

They were fed with a formula of constant composition (Table I) given in six equal portions at 7 and 10 A. M., 1, 4, 7, and 10 P. M. With a vitamin supplement [1] this and similar formulas have sufficed as the sole nutriment in a variety of studies (3), some lasting as long as eight months. The present group of patients took the formula during the six to eight weeks of this study, and then continued with the feeding for an additional 14-week period of weight reduction. Water was available without restriction.

[1] Unicap® (Upjohn), manufacturer's analysis as follows: vitamin A—5000 USP units, vitamin D—500 USP units, ascorbic acid—37.5 mg., thiamine hydrochloride—2.5 mg., riboflavin—2.5 mg., pyridoxine hydrochloride—0.5 mg., calcium pantothenate— 5.0 mg., nicotinamide—20 mg., folic acid—0.25 mg., vitamin B_{12}—1 μg.

TABLE I

Composition of diet

	Weight (gm.)	P (gm.)	F (gm.)	C (gm.)	Heat of combustion* (cal.)	"Available calories"† (cal.)	Na (mEq.)
Evaporated milk (The Borden Co.)	525.0	35.8	41.5	51.0	725	714	21.8
Corn oil (Corn Products Refining Co.)	37.5	—	37.5	—	353	332	—
Dextrose (USP)	103.5	—	—	103.5	387	381	—
Water to	1,000.0						
Total		35.8	79.0	154.5	1,465	1,427	21.8

* Specific heat of combustion as calculated (1.38 cal. per gm.) from the results of direct calorimetry. The calorimetric value for evaporated milk was obtained from The Borden Co.; those for corn oil and dextrose were taken from International Critical Tables, New York, McGraw-Hill, 1933.

† "Available calories" computed from the physiological energy factors given by McLester and Darby (6).

During the first period, lasting two to three weeks, the amount of formula was adjusted approximately to maintenance value for each patient. Exact adjustment was unnecessary since the value served only as a baseline for the subsequent alternation of caloric excess and deficit in consecutive four-day periods. During the periods of excess, 55 gm. of formula was added to each of the six feedings (485 cal. per day), and during the four days of deficit 55 gm. was subtracted from the baseline amount, making the average intake over an eight-day cycle equal to the baseline value. Three of the patients (A, B, E) started with an increment of formula in the initial period, and two (C, D) with a reduction; all of them completed four cycles.

Statistical analysis (described in the Appendix) followed standard methods for analysis of variance which make it possible to divide the variations of weight into three independent components: 1) A trend due to inequality of average caloric intake and expenditure; 2) forced oscillations, correlated with the increases and decreases of dietary intake; and 3) residual fluctuation, due to any changes in water balance not correlated with the caloric variations and to errors of measurement. The standard error of estimate of course depends upon the relative amplitudes of forced oscillation and random fluctuation; and, in principle, it can be reduced to any desired figure by a suitable choice of the number and length of cycles and the magnitude of caloric variation. In the present work the choice of four cycles of eight days each and a caloric variation of ± 485 cal. per day represented a compromise between a desire for maximal precision and the necessity of keeping the length of the experiment within practical bounds.

RESULTS

The data are summarized in Table II. The column headed *Average Intake* shows the number of calories per day given during the preliminary

TABLE II

The caloric value of labile body tissue in obese subjects

Patient (RIH No.)	Age (years)	Obesity* $\left(\frac{\text{mean wt.}}{\text{normal wt.}}\right)$	Mean weight (kg.)	Average intake (cal./day)	Weight trend (gm./day)	Caloric equivalent (cal./gm.)	Expenditure	
							Total (cal./day)	Per kg. (cal./kg. day)
A 14662	59	1.93	115.7	2,867	+21.2 (±3.4)	2.16 (±0.26)	2,820 (±9)	24.4 (±0.08)
B 14622	45	1.78	117.3	2,867	+1.6 (±5.3)	2.86 (±0.68)	2,862 (±16)	24.4 (±0.13)
C 14676	44	2.34	154.1	2,999	−44.2 (±4.9)	2.56 (±0.51)	3,113 (±26)	20.2 (±0.17)
D 14367	32	1.59	98.3	1,985	−31.5 (±2.9)	2.46 (±0.28)	2,064 (±11)	21.0 (±0.12)
E 14389	16	1.65	102.3	2,029	−89.7 (±4.9)	3.61 (±1.01)	2,359 (±90)	23.0 (±0.88)

* "Normal weight" taken from tables of the Association of Life Insurance Directors and Actuarial Society of America, New York, 1912. Cited by Duncan, G. G., Diseases of Metabolism, Philadelphia, W. B. Saunders, 1952, p. 1107.

TABLE III

Numerical functions for analysis of the trend and the response of weight to caloric imbalance

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Cal.	0	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−
t_i	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
l_i	0	1	2	3	4	3	2	1	0	1	2	3	4	3	2	1	0	1	2	3	4	3	2	1	0	1	2	3	4	3	2	1	0

period and continued as an average during the periods of alternation. The *Weight Trend* is the slope of a linear regression on time, and shows the average daily change of weight due to inequality of caloric intake and output. The *Caloric Equivalent* is a ratio of caloric variation (485 cal. per day) to the correlated change in weight. The product of weight trend and caloric equivalent gives the average daily amount of energy deposited in the body or withdrawn; it is equal to the difference between average intake and the average *Expenditure of Energy.*

The weight curves given in Figure 1 show considerable random fluctuations of weight, especially

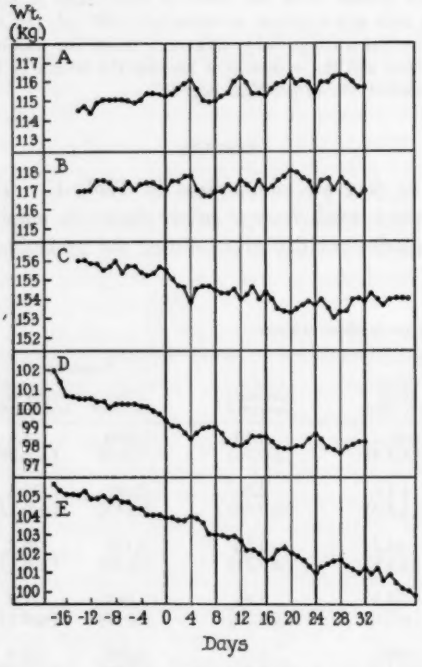

FIG. 1. DAILY WEIGHTS

The vertical lines separate the four-day periods of alternating caloric excess and deficit.

in the cases of patients B, C, and E. The caloric equivalent, being a differential coefficient, is highly sensitive to such uncorrelated changes and therefore could be estimated with a precision of only about ± 25 per cent (Table III). By contrast the average total caloric expenditure, an integral value, could be determined with a precision of better than ± 1 per cent.

The caloric equivalent for all five patients averaged 2.73 cal. per gm. (Table II). Excluding the datum of the last patient because of the discrepant value, the low precision of estimate, a weight loss during the preceding three months, and marked weight loss during the experiment, we obtain what is possibly a better estimate of the caloric equivalent for stabilized obese subjects, 2.51 cal. per gm. The difference is slight, however, and unimportant for present purposes.

The caloric expenditures expressed on a per kilogram basis fell into the range 20.2 to 24.4 cal. per kg. day. Similar values for maintenance of obese women on normal mixed diets have been reported by Harrington (4) and by Brown and Ohlson (5).

It should be noted that the changes in body weight, correlated with the caloric variations, were substantially larger than might be expected from variation of dietary sodium. The ratio of change in amount of dietary sodium to correlated change in body weight was about 37 mEq. per kg. Therefore, even if all the extra sodium were retained and diluted to form extracellular fluid, the increment would account for only one-fifth of the observed weight gain.

DISCUSSION

The caloric equivalent, as measured by alternating periods of dietary excess and deficit, sets an upper limit to the possible caloric value of labile body tissue. In the first place a part, although apparently not a large fraction, of the food energy is lost by partial absorption, excre-

tion of residues and possibly by irreversible processes in the course of storage. A calculation of "available energy" (6) suggested that about 3 per cent of the heat content of the formula might have been lost in this way (Table I).

More important as an error in the interpretation of caloric equivalent is the probable change in its value with continued excess or deficit of calories. It is well known that glycogen serves as an immediate source of energy and quickly becomes depleted. Benedict's fasting man, for instance, derived relatively little energy from carbohydrate after the third day of starvation (7). Since wet glycogen has a relatively low heat content, 1.72 cal. per gm. (8), and labile tissue protein (9) an even lower value, 0.80 (8), clearly a depletion of these substances would cause the caloric equivalent to rise. Presumably the value measured with four-day periods exceeded the true caloric equivalent of the immediate response.

The low average value found for the equivalent, 2.51 cal. per gm., shows that the primary storage of surplus energy in these obese subjects was associated with the storage of a considerable amount of water. Data in the literature make it probable that this phenomenon is not peculiar to the obese subject, but is of general occurrence. It can be estimated from the data collected by Taylor, Henschel, Mickelsen, and Keys (10), dealing with single periods of acute starvation, that normal men show a caloric equivalent of about 2 cal. per gm. during the first few days without food. Both of these values are far lower than the equivalent of about 8 cal. per gm. shown by obese subjects on reduction diets lasting for several weeks (11).

SUMMARY

1. This paper describes a method for estimating the caloric equivalent of variable weight and the average daily caloric expenditure. The basis of the method is an alternation of dietary intake between surplus and deficit to produce cyclical oscillations of body weight. On statistical analysis, the changes in weight specifically correlated with the dietary variations can be separated from a general trend and from random changes due to fluctuation of water balance and errors of measurement.

2. In the study of five obese women it appeared that the primary reservoirs of energy had a low caloric value per unit weight, probably less than 2.5 cal. per gm. This finding was interpreted as evidence that a considerable amount of water is stored and released as a part of the caloric response.

3. The daily requirement for maintenance of patients on a milk formula was 20.2 to 24.4 cal. per kg. day; values similar to these have been reported necessary for maintenance of obese women on normal mixed diets.

APPENDIX

Assume that the variations of body weight about a mean value can be represented by the sum of three independent components: a linear trend, a linear rise and fall in response to a known increase and decrease of food, and a residual fluctuation due both to uncontrolled physiological factors and to errors of measurement:

$$W_i = M + a\tau_i + b\lambda_i + \delta_i$$

where W_i is the measured body weight,

τ_i and λ_i are functions that have numerical values proportional to the day of experiment and to the stage of cycling, respectively,

δ_i is residual fluctuation, and

M, a, b are the mean, the coefficient of trend and the coefficient of response to caloric imbalance, respectively.

The numerical functions and the fluctuation term are, to a certain extent, arbitrary and can be chosen to have zero means. If this is done their properties are summarized in the following equations, of which the last expresses the independence of trend and the alternation of intake:

$$M = \frac{1}{n}\Sigma W_i, \quad \Sigma\tau_i = \Sigma\lambda_i = \Sigma\delta_i = 0, \quad \Sigma\tau_i\lambda_i = 0$$

where n is the number of observations. Minimizing the residual sum of squares, $\Sigma\delta_i^2$, in the usual way, we find the best estimates for a and b:

$$a = \frac{\Sigma W_i\tau_i}{\Sigma\tau_i^2}, \quad b = \frac{\Sigma W_i\lambda_i}{\Sigma\lambda_i^2}$$

For actual calculation, however, it is more convenient to choose integers for the numerical functions and make correction for their non-zero means. The sets t_i and l_i used in the present analysis are shown in Table III. Their values correspond to an experiment in which the first period is one of caloric surplus; when the experiment started with a deficit the series l_i was changed to correspond. In terms of the working functions:

$$a = \frac{\Sigma W_i t_i - \bar{t}\Sigma W_i}{\Sigma t_i^2 - \bar{t}\Sigma t_i} = \frac{\Sigma W_i t_i - 16\,\Sigma W_i}{2992}$$

$$b = \frac{\Sigma W_i l_i - \bar{l}\Sigma W_i}{\Sigma l_i^2 - \bar{l}\Sigma l} = \frac{\Sigma W_i l_i - 1.939\,\Sigma W_i}{51.88}$$

The residual error variance, V_e, is given by:

$$30\,V_e = \left[\Sigma W_i^2 - \frac{1}{33}(\Sigma W_i)^2\right] - \frac{1}{2992}[\Sigma W_i t_i - 16\,\Sigma W_i]^2 - \frac{1}{51.88}[\Sigma W_i l_i - 1.939\,\Sigma W_i]^2$$

and the standard errors of the coefficients by:

$$\sigma_a^2 = \frac{V_e}{2992}, \quad \sigma_b^2 = \frac{V_e}{51.88}$$

The caloric equivalent, E cal. per gm., is given by the ratio of caloric change (Δ cal. per day) and weight change (b gm. per day):

$$E = \frac{\Delta}{b}, \quad \sigma_E^2 = \frac{\Delta^2 V_e}{b^4(51.88)}$$

The maintenance requirement, R cal. per day, is obtained by subtracting a correction for the trend from the average intake, A cal. per day:

$$R = A - aE, \quad \sigma_R^2 = \frac{\Delta^2 V_e}{b^2}\left(\frac{1}{2992} + \frac{a^2}{b^2(51.88)}\right)$$

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MECHANISM OF INCREASED RENAL SODIUM EXCRETION FOLLOWING MANNITOL INFUSION IN MAN

By T. FRANKLIN WILLIAMS,[1] WALTER HOLLANDER, JR.,[1] MAURICE B. STRAUSS, ELSIE C. ROSSMEISL, AND REGINA McLEAN

(From the Medical Service and Research Laboratory, Boston Veterans Administration Hospital, The Department of Medicine, Boston University School of Medicine, and the Department of Medicine, Tufts College Medical School, Boston, Mass.)

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Mannitol, when given intravenously to normal human subjects or animals, shares with a number of other substances the property of promoting an increased rate of renal excretion of sodium, chloride, and water. The generally accepted hypothesis concerning the mechanism of this action is that mannitol exerts its effect directly on the renal tubules. The osmotic pressure of the mannitol within the tubular lumen is considered to interfere with the reabsorption of water. Sodium excretion is thought to be increased in the presence of the increased amounts of water in the tubule per unit time, either because reabsorption of sodium is limited by a maximum concentration difference for sodium which can be achieved between the tubular urine and the extracellular fluid (1–3) or because the contact of the sodium with the tubule cells is so brief that reabsorption is incomplete (4, 5). Evidence for a direct effect of mannitol on the renal excretion of salt and water is afforded by the experiments of Goodyer and Glenn (5). They injected large mannitol loads directly into one renal artery of dogs and produced prompt increases in salt and water excretion on the injected side. However another mechanism of action of mannitol in the intact animal is not excluded by these observations. Seldin and Tarail (6) have suggested that mannitol infusions may exert their effects outside the kidney by producing an expansion of extracellular volume and, when given in hypertonic solution, a contraction of intracellular volume, and thus indirectly altering some regulatory mechanism which increases the renal excretion of salt. This hypothesis has not thus far been excluded, and indeed was suggested as the probable mechanism of action of sodium chloride infusions in increasing renal sodium excretion by Green and Farah (7). They believe that the increased sodium intake influences sodium excretion primarily by establishing an osmotic pressure difference between extra- and intracellular fluids.

In most studies (1, 4, 6, 8, 9) hypertonic mannitol solutions (usually 25 per cent) have been injected. Such solutions would be expected, and have been calculated, to expand extracellular volume and contract intracellular volume. West, Kaplan, Fomon, and Rapoport (10) gave hypotonic mannitol solutions to hydrated dogs and found increased salt excretion. Here extracellular volume was expanded and although under such circumstances an absolute contraction of intracellular volume would appear unlikely, the calculated osmolarity of the plasma did rise during the mannitol infusion and therefore a relative decrease in intracellular water cannot be excluded.

Goodyer and Seldin (11) gave hypertonic mannitol solutions to two subjects during quiet standing and found less increase in salt excretion than normally occurs; to one standing, hydrated subject they gave a small amount (one liter) of hypotonic mannitol and found no increase in salt excretion.[2] Recently it has also been shown that dogs on a low salt intake prior to mannitol infusion exhibit far less natriuresis than those on a high salt intake (12). These findings suggest that other factors which affect the renal reabsorption of sodium may affect sodium excretion during mannitol diuresis.

[1] Present address: Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina.

[2] In the experiment with hypotonic mannitol, the glomerular filtration rate of the subject was about 25 per cent lower throughout the experiment—lying and standing—than is recorded for the same subject in other experiments.

In an attempt to test further the possible role of volume changes in mannitol-induced saluresis, advantage has been taken of a previous demonstration in this laboratory that the infusion of two liters of hypotonic sodium chloride—sodium bicarbonate solution into normal, *sitting*, water-loaded subjects produces only slight increases in salt excretion (13). This is therefore a situation in which expansion of extracellular volume by an infused solution should not in itself significantly change salt excretion. In the experiments described here, a similar volume of an isotonic or slightly hypotonic mannitol solution has been administered to normal, sitting, water-loaded subjects. The resulting marked increase in salt excretion, when compared to the above control situation, indicates that mannitol induces saluresis by means other than expansion of extracellular volume. Since cellular overhydration was present throughout and the mannitol infusion did not raise extracellular fluid osmolarity contraction of intracellular volume can also be excluded from consideration.

METHODS

The subjects were three male adults admitted for the study of minor complaints and free from significant physical abnormalities. The procedure was the same as that used in the previous study in which hypotonic saline-bicarbonate was infused (13) as follows: one to two hours after a breakfast low in salt, an "arterialized" blood sample was withdrawn; the subject voided and was weighed and then imbibed 1.5 L. of tap water during the next one-half to one hour. The subject remained seated for the duration of the experiment except for standing to void and be weighed every half hour. After each weighing, he drank sufficient water to maintain body weight 1.5 Kg. greater than the initial weight. After a satisfactory water diuresis had been in progress for two hours, and had been reasonably constant for at least two periods, another blood sample was withdrawn and an intravenous infusion of two liters of a five per cent solution of mannitol, U.S.P., in water (270 to 275 mM per L. by analysis) was commenced at a rate of approximately 20 ml. per min. The ingestion of water was continued so that at the conclusion of the infusion the body weight was maintained 3.5 Kg. above the initial weight.[3] Blood was withdrawn at the conclusion of the infusion and again approximately two hours later, when the experiment was terminated. In one subject, R. H., the experiment was repeated a week later, using an infusion of the control solutions, *i.e.*, two liters of a solution containing by analysis 112 mEq. sodium, 90 mEq. chloride, and 22 mEq. bicarbonate.

Analytic methods and formulae for calculation of changes in plasma volume were identical to those described in a recent report from this laboratory (14) except that serum proteins were done by the biuret reaction (15). Endogenous creatinine clearances were used as indices of rate of glomerular filtration. Mannitol analyses were made by the method of Smith, Finkelstein, and Smith (16) as modified by Elkinton (17). A number of specimens were also analyzed by a modification of the chromotropic acid method of MacFadyen (18). The results were in good agreement with those obtained by the Elkinton method. Serum and urine osmolal concentrations were determined with a Fiske freezing-point osmometer (Fiske Associates, Inc., 44 Bromfield St., Boston 8, Mass.). The machine was calibrated at the time of each set of analyses. Calibrations were made with sodium chloride solutions, with corrections for the activity of sodium chloride (International Critical Tables, 1928, Vol. IV) in such a way that a reading on the Fiske scale of "1000 milliosmols per liter" represents a freezing-point depression of 1.86° C., "100 milliosmols per liter" represents a depression of 0.186° C., etc. The calibra-

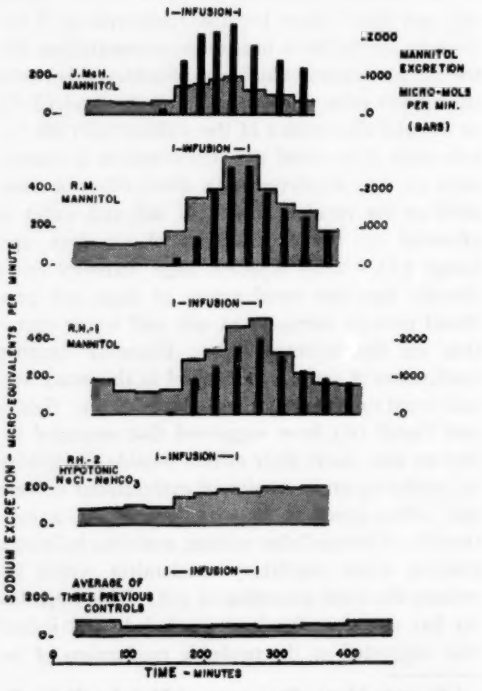

FIG. 1. THE EFFECT OF INFUSION OF 5 PER CENT MANNITOL OR OF HYPOTONIC SODIUM SALTS ON THE RENAL EXCRETION OF SODIUM AND MANNITOL

[3] Subject J. McH. experienced fullness and some nausea near the end of the infusion and his total water load was allowed to decline slowly thereafter.

TABLE I

The effect of intravenous infusion of a five per cent solution of mannitol on the constituents of the blood and upon the urinary excretion of water and solutes in water-loaded seated subjects

Subject Age Wgt., Kg. Infusate	Period*	Time Min.	Urine						Serum†					Blood†		$PV_a/PV_1 \times 100$	
			Flow ml./min.	Na micro-equiv./min.	Cl micro-equiv./min.	Mann. micro-mol./min.	Tot. solutes micro-osmols./min.	C_{cr} ml./min.	Na mEq./L.	Cl mEq./L.	Mann. m mol./L.	Total solutes mOsm./L.	T.P. gm. %	Hgb. gm. %	Hct. vol. %	From hct. & hgb.	From T.P.
J. McH. 24 61.72 Mannitol 5%	I	31	13.9	73	151		777	129	140.0	104.9	—	286	6.25	15.3	49.8	100.0	100.0
	II	32	15.5	72	134		727	121									
	III	23	15.4	66	126		767	124									
	IV	29‡	14.3	75	127	120	816	112	133.0	102.0	—	272	5.87	14.6	48.7	107.3	106.4
	V	25‡	20.3	150	217	1,550	2,440	107									
	VI	21‡	21.9	162	216	2,180	3,040	107									
	VII	20‡	22.8	184	243	2,260	3,230	104									
	VIII	24‡	23.6	199	264	2,410	3,410	115									
	IX	28	20.2	134	212	1,810	2,680	110	120.6	94.5	23.6	276	5.53	13.6	44.8	120.8	113.0
	X	39	18.9	105	166	1,450	2,280	112									
	XI	28	15.4	77	129	1,210	1,860	121	128.9	100.9	13.6	280	6.08	14.9	46.6	109.4	102.7
R. M. 27 63.20 Mannitol 5%	I	39	17.3	117	160		1,000	170	140.7	102.8	—	285	7.64	12.6	42.1	100.0	100.0
	II	39	16.2	109	156		1,390	162									
	III	39	16.3	118	159		1,040	186									
	IV	28‡	17.8	179	230	188	1,480	180	135.0	100.6	—	271	7.85	12.7	42.5	99.2	97.4
	V	27‡	24.8	360	403	733	2,970	161									
	VI	21‡	27.8	407	477	2,030	3,640	154									
	VII	22‡	34.5	568	606	2,630	4,550	167									
	VIII	24‡	35.0	579	596	2,680	4,550	162									
	IX	26	28.3	407	414	1,920	3,370	168	125.0	90.6	20.8	267	7.47	12.2	41.3	104.6	102.2
	X	25	26.8	329	344	1,530	2,790	160									
	XI	27	26.5	285	279	1,260	2,360	174									
	XII	28	21.4	183	187	896	1,760	166	132.0	93.0	7.2	257	7.27	12.2	41.3	104.6	105.0
R. H. No. 1 40 66.90 Mannitol 5%	I	26	13.7	195	194		887	113	138.1	102.6	—	288	7.43	16.1	47.0	100.0	100.0
	II	30	14.2	137	173		892	129									
	III	33	13.0	129	158		869	115									
	IV	28‡	12.7	134	156	76	900	114	132.2	98.6	—	276	7.41	15.2	45.2	109.5	100.3
	V	30‡	18.7	301	297	960	2,150	106									
	VI	29‡	21.6	351	378	1,320	3,170	102									
	VII	28‡	20.7	458	484	1,760	8,360	113									
	VIII	31‡	24.7	520	490	2,050	6,740	108									
	IX	29	10.9	338	368	1,540	4,140	113	120.3	87.2	23.2	276	7.06	15.0	44.8	111.8	105.3
	X	30	9.2	226	293	1,180	2,170	106									
	XI	29	14.3	170	244	980	3,960	108									
	XII	31	17.7	165	197	880	1,830	122	124.2	91.3	10.2	267	7.29	15.4	45.9	106.5	102.0
R. H. No. 2 40 67.02 Hypotonic NaCl–$NaHCO_3$	I	30	12.0	89	149		876	110	140.3	100.4	—	293	7.35	14.9	46.5	100.0	100.0
	II	30	12.8	92	130		845	114									
	III	28	12.9	104	132		849	108									
	IV	37‡	11.2	95	117		793	109	132.3	97.5	—	277	7.33	14.8	46.0	100.9	100.3
	V	27‡	12.4	155	158		906	115									
	VI	31‡	14.5	182	176		997	109									
	VII	27‡	15.5	199	200		1,040	113									
	VIII	30	15.5	191	196		1,050	117	133.5	97.7	—	271	6.68	14.4	41.5	109.3	110.0
	IX	30	16.0	209	197		1,020	124									
	X	29	15.2	207	183		956	115									
	XI	29	15.7	212	180		1,067	119	133.8	98.2	—	271	6.62	14.3	40.6	115.5	111.0

* First period began 30 to 50 min. after completion of ingestion of 1.5 L. water load.
† Initial serum and blood values were determined prior to water loading; second and third determinations, at beginning of indicated periods; fourth determinations, at end of experiment.
‡ Periods of intravenous infusions. Infusion began approximately half-way through the first indicated period.

tion has been checked with sucrose solutions of known molality and found to be accurate to ± 2 per cent of the scale reading. Results are recorded for simplicity as "mOsm. per L." (Table I), although they are actually not determinations of osmolar concentration but of osmolal concentration, *i.e.*, milliosmols per kilogram of serum water or urine water. No interpretation is made about the degree to which any given solute contributes to the total freezing-point depression.

RESULTS

The rate of urine flow and solute excretion, endogenous creatinine clearances, serum and blood analyses, and calculated changes in plasma volumes are given in Table I. Potassium analyses, though performed on all samples, are not included in the table because no changes of note in potassium concentration in plasma or its urinary excretion rate were observed.

As recorded in Table I and as shown graphically in Figure 1, the rate of sodium excretion began to increase promptly with the beginning of the mannitol infusion. The peak rate of sodium excretion in every subject was three to five times the pre-infusion rate. In every subject the rate of sodium excretion closely paralleled the rate of mannitol excretion, during the infusion periods when mannitol and sodium excretion were increasing and during the post-infusion periods as well, when the excretion of both declined almost exponentially. Figure 2 demonstrates for the post-infusion periods in each subject that a straight-line relationship appears to exist between the rates of excretion of the two substances. The rate of excretion of sodium was not as well correlated with the total osmotic load in the urine (mOsm. per min.), with urine volume, or with mannitol concentration in the urine.

The results of infusing equal volumes of hypotonic sodium chloride-bicarbonate solution, as shown in the two lower graphs of Figure 1, were quite different. In the one such experiment reported here, R. H. No. 2, there was an increase in sodium and chloride excretion, but this increase began more slowly, was not as large as the in-

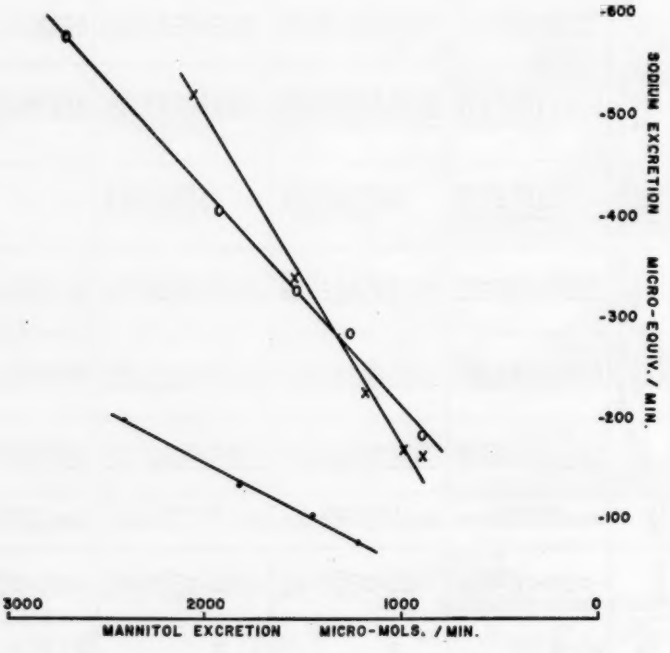

FIG. 2. RELATIONSHIP BETWEEN MANNITOL AND SODIUM EXCRETION FOLLOWING INFUSION OF 5 PER CENT MANNITOL

Open circles, R. M., closed circles, J. McH., crosses, R. H. No. 1.

crease in sodium excretion which accompanied mannitol infusion in the same subject, and continued at a fairly constant rate for at least two hours after the infusion was completed. In the three control subjects previously reported, whose results are averaged in Figure 1 because they were so similar to each other, little increase in sodium excretion occurred during or after the chloride-bicarbonate infusion in the sitting position.

Changes in plasma volume during the infusions, shown in Table I, and changes in chloride space, calculated by the formulae of Elkinton and Winkler (19) showed increases with the mannitol infusions that were slightly less than those found following the control infusions. In the mannitol experiments reported here, the maximum calculated increase in chloride space was 7 to 11 per cent; in the control experiment on R. H., the increase was 12.7 per cent; in the previously-reported controls, the increase was 12 to 16 per cent. This might be considered paradoxical since 550 milliosmols of mannitol were injected as contrasted to the 448 milliosmols of the sodium solution in the control studies. However the excretion of mannitol was so rapid that the maximum amount in the body at any time was less than 370 milliosmols. This maximum value obtained at the conclusion of the infusion by which time a minimum of 180 milliosmols of mannitol had already been excreted in the urine.

The total solute concentrations of the sera, estimated by freezing-point depression, did not change materially in any subject as a result of the mannitol infusion; see Table I.

The endogenous creatinine clearance exhibited no noteworthy changes throughout these experiments. During the mannitol infusion the serum sodium concentration fell 10 to 13 mEq. per L. in every subject, and rose again in the post-infusion periods. The filtered load of sodium was therefore falling during the time in which sodium excretion was rising, and rising when the sodium excretion was falling.

At the peak of the mannitol diuresis, urine flows had increased 8 to 10 ml. per min. above the initial flows. The highest flow recorded, 35 ml. per min., represented 21.7 per cent of the filtered water. The urine flow only roughly paralleled the rate of mannitol, sodium or total solute excretion. In Expt. R. H. No. 1 there appears to be an antidiuresis despite the maintained water load, as may be seen from the fall in urine flow and rise in solute concentration in periods IX and X.

Associated with the large increase in rate of total solute excretion during the mannitol infusions, there was a rise in the total solute concentration in the urine. The "free water clearance," C_{H_2O}, which is a measure of the degree to which solute reabsorption in excess of water reabsorption has taken place in the renal tubule, has been calculated by Wesson and Anslow's equation, $C_{H_2O} = \left(1 - \frac{U_{osm}}{P_{osm}}\right) V$ (20). The maximal value of free water clearance attained by two subjects during mannitol diuresis was no higher than the maximal value attained during the preceding periods of water diuresis alone. The third subject, R. M., showed a free water clearance of 18.0 ml. per min. during the mannitol infusion, in contrast to a maximal value of 13.7 ml. per min. during the preceding water diuresis.

DISCUSSION

It is unlikely that the greatly increased salt excretion induced by mannitol infusion in these experiments was due entirely to changes in extracellular volume since an equal or greater expansion of extracellular volume by a hypotonic salt infusion in the control experiments was associated with relatively small increases in salt excretion.

Furthermore, in every subject the sodium excretion began rising promptly in the first collection period after the mannitol infusion was begun, at a time when the extracellular volume had been increased by less than a liter, and by less than 500 ml. in subject R. M. Similarly the excretion of sodium began to decline promptly with the decline in mannitol excretion, at a time when changes in extracellular volume between successive periods must have been very small.

Seldin and Tarail as well as Green and Farah suggested that cellular dehydration, as a result of elevated extracellular osmolarity, might stimulate sodium excretion. This possibility is excluded in the present experiments both because of the maintained state of overhydration and because the serum osmolarity was unchanged by the isotonic or slightly hypotonic mannitol infusions (see Table I).

Although the water turnover was greater in the mannitol than in the control experiments (*i.e.*, the rate of water ingestion and of water excretion was greater) there was never a larger water load retained in the body such as Leaf, Bartter, Santos, and Wrong (21) have associated with increased urinary electrolyte excretion.

It seems most likely that the mannitol exerts its saluretic effect in the experiments in normal human subjects recorded here by a direct renal tubular action. The rate of renal excretion of sodium appears to be directly proportional, under the conditions of these experiments, to the rate of excretion of the mannitol, which in turn is directly proportional to the filtered load of mannitol (22). However, it will be noted that the slopes and intercepts of the lines relating mannitol and sodium excretion (Figure 2) are not identical in the three subjects, indicating that other factors than the mannitol excretion rate are active in regulating sodium excretion, as has been observed previously (11, 12).

Mannitol, as a non-electrolyte which probably does not cross cell membranes in significant amounts, would be expected to affect the equilibria of diffusible electrolytes as well as water across the cell membranes which are in contact with the mannitol. Although this effect will be greatest where the mannitol concentration is highest, *i.e.*, in some portion of the renal tubule, it will theoretically be true of any body cell. The actual effect of mannitol solutions on body cells remains to be investigated.

In two subjects, as noted in the results, solute reabsorption without water reabsorption (free water clearance) apparently reached a maximal rate, uninfluenced by rising solute loads. As the rate of solute excretion rose, the urine solute concentration necessarily rose. In the third subject, higher free water clearances were associated with the increased solute loads of the mannitol diuresis. A similar finding has been noted and discussed by Welt, Young, Thorup, and Burnett (23). It should be borne in mind that such changes could always be due to variations in endogenous anti-diuretic hormone secretion, a factor which cannot be adequately excluded by present methods. Evidence of varying anti-diuretic hormone activity in Experiment R. H. No. 1 was noted in the results.

SUMMARY AND CONCLUSIONS

The intravenous infusion of an isotonic or slightly hypotonic solution of mannitol in sitting, water-loaded subjects leads to a marked increase in the urinary excretion of sodium and chloride although the filtered load of sodium presented to the renal tubules is actually diminished.

Since comparable expansion of extracellular fluid volume by hypotonic sodium solutions fails to produce similar increases in sodium and chloride excretion, it appears unlikely that the mannitol effect derives from expansion of extracellular fluid volume. In view of the fact that cellular overhydration was already present before the mannitol infusion was commenced, and the fact that isotonic or slightly hypotonic mannitol infusion does not increase extracellular osmolarity, cellular dehydration does not seem to be a factor.

The observations lend support to the hypothesis that the main site of action of mannitol (and allied materials) in increasing urinary sodium and chloride excretion is in the renal tubule.

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A FAMILIAL HEMORRHAGIC TRAIT ASSOCIATED WITH A DEFICIENCY OF A CLOT-PROMOTING FRACTION OF PLASMA[1]

By OSCAR D. RATNOFF and JOAN E. COLOPY

(From the Department of Medicine, the Western Reserve University School of Medicine, and University Hospitals, Cleveland, Ohio)

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Recently, three patients have been studied in whom the clotting time of venous blood was greatly prolonged. None of the patients had significant hemorrhagic symptoms. The trait was apparently familial and appeared in both sexes. The patients' disorder could not be identified with any known bleeding disease. However, in each case, the plasma was deficient in a substance found in normal globulin which accelerated the clotting of normal, platelet-deficient plasma.

This clot-promoting fraction, present in heated, barium sulfate-adsorbed serum, was neither thrombic nor thromboplastic and did not alter the rate of conversion of fibrinogen to fibrin by thrombin, nor of prothrombin to thrombin by tissue thromboplastin. By inference, its effect was upon an earlier stage of clotting, presumably upon the development of thromboplastic activity in shed blood. However, the fraction did not appear to contain any of the components of plasma known to be necessary for the optimal evolution of plasma thromboplastic activity. The characteristics of the clot-promoting fraction, deficient in the plasma of these three patients, suggests that its properties may be identical with those of an unidentified accelerator of clotting described in an earlier study (1). Tentatively, until its mode of action becomes clear, this fraction may be named Hageman factor, after Patient I.

CASE REPORTS

I. J. R. H. (Crile V.A.H. No. 65930), a 37-year-old railroad brakeman, was first studied during July, 1953, because a prolonged clotting time was observed during routine pre-operative studies. He had no history of hemorrhagic difficulties, purpura or anemia, and had not bled after a tonsillectomy when he was 6 years old, after an injury to the hand at 10, nor after a dental extraction, at 36. He had had symptoms and laboratory evidence of a duodenal ulcer since 1943, and was to be operated upon for the relief of pyloric obstruction, but this procedure was not carried out because of the abnormal clotting time. A few months before he was studied, he had had black stools for 2 days, but he was taking bismuth orally at the time. There was no familial history of bleeding, and, to his knowledge, the patient was unrelated to Patients II and III. His platelet count, clot retraction, and bleeding time were normal for the methods used. A tourniquet test was strongly positive. Innumerable petechiae appeared on the forearm below a blood pressure cuff inflated at 100 mm. Hg pressure for 5 minutes.

II. M. R., a 46-year-old housewife, was first studied during July, 1954, because of a prolonged clotting time. She first learned that her clotting time was abnormal in 1938, when a right salpingo-oophorectomy was postponed for 2 hours on this account. The operation was performed without difficulty. She had no history of hemorrhagic symptoms, menorrhagia or abnormal bleeding at the birth of her son in 1936 and had no abnormal bleeding after an appendectomy at 30 years of age, a hemorrhoidectomy at 31, nor several dental extractions. Patient III is one of this patient's two surviving sisters. Five other relatives, including the son of Patient II and her third sister, do not have an abnormal clotting time. The patient had severe varicose veins and a few tiny ecchymotic spots on her legs. The platelet count, clot retraction and bleeding time were all normal. The tourniquet test was equivocal. About 50 petechiae appeared in an area under the cuff, about 50 at the crease of the elbow, and 6 in an area the size of a silver dollar on the volar surface of the forearm, after a blood pressure cuff was inflated at 100 mm. Hg for 5 minutes. By electrophoretic analysis, her plasma contained 3.44 grams of albumin, 0.40 grams of alpha globulin, 0.68 grams of beta globulin, 0.72 grams of gamma globulin, and 0.35 grams of fibrinogen-like material per 100 ml.

III. T. R., a 50-year-old housewife, was studied in July, 1954, in a survey of members of the family of Patient II. She had never had any symptoms suggestive of a bleeding tendency, and was delivered of 4 daughters, had several dental extractions and a minor operation upon her breast, all without difficulty. As a child, one of the patient's daughters had nosebleeds, and this child's

[1] This study was supported in part by a research grant (P.H.S. No. H1661C) from the National Heart Institute of the National Institutes of Health, Public Health Service, and in part by a grant from the Cleveland Area Heart Society.

daughter, now 6, also has nosebleeds, but neither of these two individuals has the coagulative defect.

MATERIALS

Blood was drawn from the antecubital veins of the patients and of normal human adults, using No. 18 gauge needles coated with tris(2-hydroxyethyl)dodecyl-ammonium chloride (Armour Needle Coating Solution) and glass syringes coated with silicone (Desicote, Beckman).

Oxalated plasma was prepared by mixing nine parts of venous blood with one part of 0.1 M sodium oxalate solution, and removing the cells by centrifugation at 2500 rpm for 15 minutes at room temperature in an International SB Size 1 centrifuge. This plasma was kept in ice water and used within a few hours, or stored at 4°C. or −27°C. for as long as a year until used.

Platelet-deficient "native" plasma was prepared without the addition of an anticoagulant by the method of Conley, Hartmann, and Morse (2). Venous blood was centrifuged at 10,500 rpm (14,000 G) for 10 minutes in silicone-coated lusteroid tubes, in a Servall SS-1 angle centrifuge refrigerated at −5°C. The upper portion of the plasma was removed with a silicone-coated pipette and kept until used in silicone-coated lusteroid tubes immersed in ice water. Such native plasma contained less than 50 platelets per $mm.^3$, and, rarely, a few erythrocytes.

Lipid-deficient serum was prepared at 4°C. by adding 10 ml. of pooled human serum drop-wise to 200 ml. or more of a mixture of 2 parts of chloroform and 1 part of absolute methyl alcohol, in the Monel metal cup of a Waring blender rotating at moderate speed. The mixture was filtered through Whatman No. 1 filter paper in a Büchner funnel. The residue on the paper was dried at 4°C. in a desiccator containing concentrated sulfuric acid and soda-lime, using suction, and then ground with buffer in a volume equal to that of the original serum, using a mortar and pestle. Remnants of paper were removed by centrifugation at 10,500 rpm for 3 minutes in a Servall SS-1 centrifuge at −5°C. The supernatant solution of lipid-deficient serum had about half the concentration of protein of the original serum.

Barium sulfate-adsorbed plasma or serum was prepared by incubating a mixture of oxalated plasma or serum and one-tenth volume of powdered barium sulfate (Baker) at 37°C. for 10 minutes, stirring occasionally. The adsorbed plasma or serum was then separated by centrifugation. Adsorbed plasma was presumably deficient in prothrombin, proconvertin and plasma thromboplastin component. Adsorbed serum lacked, in addition, fibrinogen, proaccelerin and anti-hemophilic factor.

A *globulin fraction* of plasma or serum was prepared by dilution with water and acidification to approximately pH 5.3. In some experiments, this crude globulin was prepared from native plasma in silicone-coated glassware in such a manner that at no time did it come in contact with an anticoagulant or with a glass or metal surface (3). The precipitated globulin was dissolved in buffer, usually in a volume equal to that of the original sample of plasma. Such *native plasma globulin* is known to contain many substances, including prothrombin, fibrinogen, proaccelerin, proconvertin, anti-hemophilic factor, plasma thromboplastin component, and small amounts of calcium (3), as well as an unidentified accelerator of clotting described previously (1).

In other experiments, the globulin fraction of serum or of oxalated plasma was prepared in Pyrex tubes by dilution of the sample with 19 volumes of water and acidification to approximately pH 5.3. When the sample was 1.0 ml. in volume or larger, one per cent acetic acid was used. When samples of 0.1 ml. of oxalated plasma were used, the acidification was arbitrarily performed by the addition of 0.1 ml. of 0.25 per cent acetic acid. The globulin thus prepared was separated by centrifugation at room temperature for 15 minutes at 2500 rpm and dissolving the precipitate in buffer.

Further fractionation of the globulin was performed by precipitation with ammonium sulfate. The globulin was concentrated either 2 or 4 times compared with the original plasma or serum. The globulin solution was diluted with ammonium sulfate solution which was saturated at 4°C. and neutralized with concentrated ammonium hydroxide. Successive fractions were prepared by the addition of sufficient ammonium sulfate solution to raise the concentration to the desired degree of saturation. Each mixture was refrigerated at 4°C. for 24 hours and the precipitate separated by centrifugation at room temperature. The precipitates were dissolved in water, reprecipitated by ammonium sulfate for 24 hours and recentrifuged. The precipitates were then dissolved in water and dialyzed in Visking No-Jax cellophane casings, first against running tap water and then against frequent changes of buffer. The supernatant fluid remaining after half-saturation with ammonium sulfate was dialyzed in the same way. The fraction precipitated between 33 and 50 per cent saturation with ammonium sulfate from the globulin of serum which had been stored at 4°C. for 6 weeks and then adsorbed with barium sulfate and heated at 56°C. for 30 minutes will be designated the *clot-promoting fraction.* However other fractions also possess considerable clot-promoting activity; the method described was *not* designed to result in a maximal yield.

Platelets were prepared from Patient I's blood by mixing 10 ml. of blood with 0.3 ml. of 5 per cent sequestrene Na2 in a silicone-coated centrifuge tube.[2] The blood was centrifuged for 20 minutes at 800 rpm in an International SB Size 1 centrifuge. The platelet-rich plasma was then recentrifuged at 2500 rpm for 15 minutes. The platelets were suspended in 5 ml. of 0.15 M sodium chloride solution and recentrifuged, a total of three times, and finally suspended in 2 ml. of sodium chloride solution.

The *buffer* solution consisted of 7.30 grams of sodium chloride, 2.76 grams of barbital and 2.06 grams of sodium barbital, diluted to a volume of 1 liter with distilled

[2] Sequestrene Na2 was provided through the courtesy of the Alrose Chemical Company, Providence.

water. The buffer was thus composed of 0.025 M barbital and 0.125 M sodium chloride, at pH 7.5.

METHODS

The clotting time of whole blood was measured by a modification of the method of Lee and White (4), using dry Pyrex or silicone-coated tubes (internal diameter, 11 mm.) (5). In 107 normal individuals, the clotting time at 25°C. in Pyrex tubes averaged 32.1 minutes (range, 11 to 56; standard deviation of the mean, 9.0 minutes). In silicone-coated tubes, the clotting time in 72 normal persons averaged 98.6 minutes (range, 55 to 165; standard deviation of the mean, 19.2 minutes).

The *recalcified clotting time* of oxalated plasma was determined, usually at 25°C., in Pyrex tubes (internal diameter, 8 mm.). In duplicate, 0.2 ml. of plasma and 0.2 ml. of 0.025 M calcium chloride solution were mixed in tubes immersed in ice water and then rapidly transferred to a water bath at 25°C. The clotting time was determined by tilting both tubes at 15- or 20-second intervals. After 20 or 30 minutes, the tubes were observed at progressively longer intervals. The clotting time was the period which elapsed from the time that the tubes were transferred to the 25°C. bath until the appearance of a solid clot. The longer of the duplicate clotting times is recorded. However, it is noteworthy that the contents of the duplicate tubes almost always coagulated within 30 seconds of each other when the clotting time was 12 minutes or less. In 63 normal subjects, the recalcified clotting time at 25°C. averaged 6.9 minutes (range, 3 to 12; standard deviation, 2.0 minutes).

The *clotting time of native plasma* was measured at 37°C. by transferring 1 ml. of plasma to each of 3 Pyrex and 3 silicone-coated tubes (internal diameter, 11 mm.). The tubes were closed with rubber stoppers and incubated in a water bath. The clotting time was determined in the same manner as that of whole blood (5). Native plasma obtained from normal individuals usually clots in 60 minutes or less in Pyrex tubes (2). In silicone-coated tubes, the clotting time is much longer, and normal plasma may not clot at all (2).

The clotting time of native plasma was also tested in Pyrex tubes containing weighed amounts of *crushed Pyrex glass (50 to 100 mesh)*, which had been washed with chromic acid and thoroughly rinsed with distilled water.

The conditions under which the effect of various substances upon the clotting time of whole blood, recalcified oxalated plasma, or native plasma were tested will be described with each experiment. In general, all mixtures were prepared in tubes immersed in mixtures of ice and water, and then transferred to water baths at 25 or 37°C.

The possible presence of a *circulating anticoagulant* was tested in native plasma by the method of Conley, Hartmann, and Morse (6), and in oxalated plasma, by measuring the recalcified clotting time of suitable mixtures of the patient's plasma and normal plasma.

The *thrombin time*, that is, the clotting time of an equal mixture of oxalated plasma and bovine thrombin (Parke, Davis and Co.) was determined at 37°C. in Pyrex tubes (internal diameter, 8 mm.) (7). The results were interpolated relative to a standard thrombin time, for normal individuals, of 30 seconds. In any given normal adult, the thrombin time rarely varied by more than 3 seconds from that of any other normal adult (7).

The *prothrombin time* was determined at 37°C. by a *one-stage method* (8), mixing 0.1 ml. of oxalated plasma, 0.1 ml. of rabbit or human brain thromboplastin or Russell Viper Venom, and 0.1 ml. of 0.025 M calcium chloride solution in Pyrex tubes (internal diameter, 8 mm.). Human or rabbit brain thromboplastin (Cappell Laboratories, Wayne, Pa.) was prepared by suspending 150 mg. in 4 ml. of 0.15 M sodium chloride solution and 0.05 ml. of 0.1 M sodium oxalate solution, and heating the mixture between 45 and 50°C. for 15 minutes. Rabbit brain thromboplastin (Difco Laboratories, Detroit) was suspended without the addition of oxalate and heated for only 10 minutes. Russell Viper Venom (Stypven, Burroughs, Wellcome) [3] was dissolved in a concentration of 0.01 per cent in water. The suspensions of thromboplastin described will be referred to as "undiluted."

A more *specific test for prothrombin* was performed by Alexander's modification (9) of the method of Owren and Aas (10) in which the concentration of prothrombin is determined in the presence of proaccelerin and proconvertin. The proaccelerin and proconvertin were provided by an equal mixture of barium sulfate-adsorbed bovine plasma and of pooled human serum which had been decalcified with sodium oxalate after storage at 4°C. for 4 months.

The *two-stage prothrombin method* of Ware and Seegers (11) was used, employing Difco reagents and bovine fibrinogen solution.[4] The fibrinogen solution was prepared by dissolving 150 mg. of dried materials (12) in 10 ml. of 0.70 per cent sodium chloride solution. The resultant solution, after filtration through Whatman No. 1 paper, contained about one per cent coagulable protein in 0.85 per cent sodium chloride solution.

The *serum prothrombic activity* was measured by allowing 2.25 ml. of blood to clot at 37°C. in a Pyrex tube (internal diameter, 8 mm.). One hour after the blood was drawn, a volume of 0.25 ml. of 0.1 M sodium oxalate solution was added, the contents thoroughly mixed with a wooden applicator stick, and the mixture centrifuged at room temperature for 15 minutes at 2500 rpm in the International centrifuge. The oxalated serum was then heated at 37°C. for 30 minutes to destroy any remaining free thrombin. Heating for shorter periods was not adequate for this purpose. The prothrombic activities of the patient's serum and oxalated plasma were then compared using the specific prothrombin method. This test gave somewhat erratic results, but in 46 normal

[3] Stypven was provided through the courtesy of Burroughs, Wellcome and Company, New York.

[4] Dr. Walter Seegers, Detroit, kindly provided the preparation of purified bovine fibrinogen.

TABLE I

The clotting time, thrombin time and plasma prothrombic activity in three patients

Test	Case I	Case II	Case III	Normal range
Clotting time, 25°C., whole blood (*min.*)				
Pyrex tubes	87	86	90	11–56
Silicone-coated tubes	300	330	300	55–165
Clotting time, 37°C., native plasma (*min.*)	∞	∞	—	<60
Clotting time, 25°C., recalcified plasma (*min.*)	102	47	47	3–12
Thrombin time (*sec.*; control = 30 seconds)	28	30	—	27–33
Plasma prothrombic activity (*sec.*; one-stage method*)				
Patient	14	17	—	—
Control	13	17	—	—

* Rabbit brain thromboplastin.

individuals, the serum prothrombic activity, one hour after blood was drawn, was 40 per cent or less in 44 instances, 35 per cent or less in 42 instances, and 30 per cent or less in 38 instances.

The *effect of trypsin* on oxalated plasma was tested in Pyrex tubes (internal diameter, 8 mm.) by mixing 0.2 ml. of plasma and 0.1 ml. of aqueous trypsin solution (Worthington Chemical Co.). The trypsin solution consisted of 0.8 mg. of dried material per ml. of water; the dried preparation contained as much as 50 per cent magnesium sulfate.

The presence of *staphylocoagulase-reacting factor* in oxalated plasma was tested by the slide method of Cadness-Graves, Williams, Harper, and Miles (13), using a 24-hour culture on tryptose blood agar base of a strain of *micrococcus staphylococcus var. aureus* known to be coagulase positive.

The presence of *plasminogen* (the precursor of plasmin, the proteolytic enzyme or enzymes of plasma maximally active against most substrates at neutrality) was tested in oxalated plasma by the addition of 0.1 ml. of a solution of streptokinase-streptodornase, containing 600 Christensen units (14) of streptokinase [5] per ml. of water, to 0.2 ml. of plasma in Pyrex tubes (internal diameter, 8 mm.). The mixture was clotted by the addition of 0.1 ml. of bovine thrombin solution (Parke, Davis and Co.) containing 10 National Institutes of Health (N.I.H.) units (15) per ml. of buffer. The clot lysis time was then measured at 37°C.

The presence of plasminogen in the clot-promoting fraction of serum was tested in Pyrex tubes by mixing 0.3 ml. of the fraction, 0.3 ml. of streptokinase solution (100 units per ml.), 0.3 ml. of bovine fibrinogen solution (one per cent coagulable protein) and 0.1 ml. of bovine thrombin solution (10 N.I.H. units per ml.). The clot lysis time was then measured at 37°C. and compared with the lysis time of the same mixture in which buffer was substituted for streptokinase or for the fraction.

[5] Varidase®, a commercial preparation of streptokinase and streptodornase, was provided through the courtesy of Lederle Laboratories, Pearl River, New York.

RESULTS

The nature of the clotting defect

In each of the three cases the *clotting time of whole blood* at 25°C. was prolonged both in glass and in silicone-coated tubes (Table I). This may have been due to a cellular or plasma defect. To test this, the *clotting time of native, cell-free plasma* from Patients I and II was measured in Pyrex tubes. Normally, the clotting time of such cell-free plasma is about that of whole blood (2). In these cases, however, native plasma was incoagulable (Table I). One must conclude that the defect was in the plasma.

The platelet-free plasma, however, was not totally incoagulable. In duplicate, one ml. of plasma of Patient I was pipetted into Pyrex tubes containing crushed Pyrex glass. The clotting time at 37°C. was 182 minutes in the presence of 100 mg. of glass and 144 minutes in the presence of 500 mg. of glass. The platelet-deficient plasma also clotted when it was diluted with 0.15 M sodium chloride solution (Table II). The minimum clotting time was noted in a mixture of 60

TABLE II

The effect of dilution on the clotting time of platelet-deficient plasma

Per cent plasma	Clotting time*
	min.
100	∞
80	280
60	113
40	144
20	∞

* Clotting time in duplicate of mixtures of Patient I's platelet-deficient native plasma and 0.15M sodium chloride solution, in Pyrex tubes (internal diameter 11 mm.) at 37°C.

per cent plasma and 40 per cent sodium chloride solution.

The *recalcified clotting time* was greatly prolonged in each instance. The plasmas from Patients I and II were not mutually corrective. In one experiment the recalcified clotting time in Case I exceeded 60 minutes, and in Case II was 30 minutes. An equal mixture of the two clotted in 41 minutes. Further evidence for the identity of the defect in all three cases will be presented subsequently.

Cases have been described in which a prolonged clotting time was attributed to a *circulating anticoagulant*. None could be demonstrated in native plasma, in Case I, nor in oxalated plasma, in all three cases. For example, the recalcified clotting time in Case II was 51 minutes, and that of a normal plasma, was 5 minutes. A mixture of 80 parts of the patient's plasma and only one part of normal plasma clotted in ten minutes. Other experiments to be described confirm the probable absence of a circulating anticoagulant.

An attempt was made to determine whether the patients' clotting abnormality could be classified among known disorders of blood clotting. The *thrombin time* and *one-stage prothrombin time* were normal in Cases I and II (Table I). Furthermore, the prothrombin time was normal whether one used tissue thromboplastin from human or rabbit brain, whether either preparation was undiluted or diluted 1:100 with 0.15 M sodium chloride solution, or whether one used Russell Viper Venom as a thromboplastin. On the other hand, a *suspension of platelets* did not have an impressive clot-accelerating effect (Table III). In Case II, the plasma prothrombic activity, by the more specific method of Owren and Aas (9, 10), was 70 per cent of one normal plasma, and 95 per cent of another. The conversion of prothrombin to thrombin during the clotting of whole blood in Pyrex tubes was tested in Cases I and II. One hour after the blood was drawn, the *serum prothrombic activity* was 43 and 63 per cent of that of the plasma in Cases I and II, respectively, slightly elevated values.

TABLE III

The effect of a platelet suspension upon the recalcified clotting time

Plasma	Clotting time*	
	Platelets	Buffer
	min.	*min.*
Patient I	32	74
Control	4	8

* Clotting time in duplicate of a mixture of 0.2 ml. of oxalated plasma, 0.1 ml. of platelet suspension or buffer, and 0.2 ml. of 0.025M calcium chloride solution, at 25°C. in Pyrex tubes (internal diameter, 8 mm.). The platelets were derived from Patient I's blood and were in a volume of buffer one-fifth that of the original blood sample.

TABLE IV

Effect of plasmas from patients with known hemorrhagic disorders upon the recalcified clotting time

	Plasma	Clotting time*
		min.
A.	Patient I, 0.2 ml.	60
	Patient II, 0.2 ml.	47
	Patient I, 0.1 ml. + Patient II, 0.1 ml.	60
B.	Patient I, 0.1 ml. + Buffer 0.1 ml.	60
	Patient I, 0.1 ml. + Normal plasma (diluted 1/160) 0.1 ml.	12
	Patient I, 0.1 ml. + Hemophilic plasma (diluted 1/160) 0.1 ml.	11
	Patient I, 0.1 ml. + Christmas disease (diluted 1/160) 0.1 ml.	10
	Patient I, 0.1 ml. + Plasma deficient in 4th thromboplastin component (diluted 1/160) 0.1 ml.	12
C.	Patient I, 0.1 ml. + Buffer 0.1 ml.	54
	Patient I, 0.1 ml. + Normal plasma (diluted 1/80) 0.1 ml.	12
	Patient I, 0.1 ml. + Patient II's plasma (diluted 1/80) 0.1 ml.	52
	Patient I, 0.1 ml. + Plasma "A.A." deficient in plasma thromboplastin antecedent (diluted 1/80) 0.1 ml.	10
	Patient I, 0.1 ml. + Plasma "W" deficient in plasma thromboplastin antecedent (diluted 1/80) 0.1 ml.	11

* Clotting time in duplicate at 25°C. of mixtures of oxalated plasma and 0.025M calcium chloride solution. A, B and C represent separate experiments. In A, 0.2 ml. of plasma were mixed with 0.2 ml. of calcium chloride solution. In B and C, 0.1 ml. of Patient I's plasma was mixed with 0.1 ml. of buffer, or a dilution of plasma in buffer, and 0.1 ml. of calcium chloride solution.

The presence of the hypocoagulative trait in both sexes makes it unlikely that the patients had either hemophilia or a deficiency of plasma thromboplastin component (Christmas disease) (16–18). Table IV illustrates the correction of the defect in Case I by very small amounts of the plasma of patients with each of these disorders. Similar observations were made for hemophilia in Cases II and III, and for Christmas disease, in Case II. In the same manner, plasma from patients thought to have a deficiency of plasma

thromboplastin antecedent [6] (19) and of a fourth thromboplastin component [7] (20) corrected the defect in Patients I and II. Similarly, as little as 0.05 ml. of oxalated plasma from patients with deficiencies of plasma thromboplastin antecedent or the fourth thromboplastin component shortened the clotting time of 1 ml. of Patient II's whole blood to the same degree as 0.05 ml. of normal oxalated plasma. One-tenth part of oxalated plasma from Patients I and II were virtually without effect on the clotting time of Patient I's whole blood.

Patient I's defect was corrected *in vivo* by the *transfusion of 250 ml. of citrated human plasma.* One and a half hours after the transfusion had been completed, the recalcified clotting time was 12 minutes, compared with a pre-transfusion clotting time of 60 minutes; 18 hours after the transfusion the recalcified clotting time was 65 minutes.

Trypsin clots blood or plasma by converting prothrombin to thrombin. It is not clear whether a co-factor is needed. Trypsin clotted the plasma in Case II in the same time as a normal control.

Human plasma is also clotted by staphylocoagulase, a product of certain staphylococci. This process is mediated through a coagulase-reacting factor in plasma. The plasma in Case II was clotted as promptly as a normal plasma by a culture of staphylococci, demonstrating that the patient's plasma contained coagulase-reacting factor.

Preliminary studies suggested that the defect in Case I could be corrected by the addition of the acid-insoluble globulin precipitated from diluted normal plasma at approximately pH 5.3. This observation made it possible to corroborate the identity of Cases I, II, and III, and to separate these cases from known instances of other hemorrhagic disorders.

One-tenth ml. of oxalated plasma to be tested was pipetted into a Pyrex tube. The acid-insoluble globulin was precipitated by dilution and acidification and dissolved in 0.1 ml. of buffer. To this solution was added a volume of 0.5 ml. of native plasma from Patient II. The clotting time of the mixture was determined at 37°C., tilting the tubes every 30 seconds.

[6] Plasmas from A. A. and W., two patients with a deficiency of plasma thromboplastin antecedent, were kindly provided by Dr. R. L. Rosenthal, New York.

[7] Plasma from C. C., the patient with a deficiency of the fourth thromboplastin component, was kindly provided by Dr. T. Spaet, San Francisco.

TABLE V

The clot-promoting effect of a globulin fraction of oxalated plasma on the native plasma of Patient II

Source of globulin	Clotting time*
	min.
Buffer solution alone	∞
Control plasma, fresh	2
Control plasma, stored 8 months	4
Plasma, Case No. 1, stored 12 months	36
Plasma, Case No. 2, fresh	48
Plasma, Case No. 3, fresh	29
Hemophilic plasma, fresh	4
Hemophilic plasma, stored 10 months	3
Christmas disease plasma, stored 13 months	4
Plasma deficient in plasma thromboplastin antecedent, stored 11 days	3
Plasma deficient in fourth thromboplastin component, stored 6 months	2
Plasma with circulating anticoagulant *vs.* thromboplastin, stored 5 months	5

* Clotting time in Pyrex tubes (internal diameter, 11 mm.) at 37°C. of mixtures of 0.1 ml. globulin solution and 0.5 ml. fresh native plasma of Patient II. The patient with a circulating anticoagulant had lupus erythematosis. The stored plasmas had been kept in the frozen state.

Representative results of this experiment are listed in Table V. Only the globulin from the three patients under study failed to shorten the clotting time dramatically.

It is of note that *plasminogen,* which could be activated by streptokinase, was demonstrable in the plasma in Case II.

Some properties of a clot-promoting factor of normal plasma or serum

The preceding experiments demonstrate that the defect could be corrected by normal plasma or a plasma globulin fraction. A further delineation of the clot-promoting activity of normal blood was attempted. Serum depleted of lipid was as corrective as normal serum. Barium sulfate-adsorbed oxalated normal plasma was almost as effective as unadsorbed oxalated plasma. On the other hand, an eluate of the material adsorbed to the barium sulfate also exhibited some activity. The defect could be corrected by normal oxalated plasma, stored at 4°C. for 12 weeks, or normal serum, stored at 4°C. for 6 weeks. The clot-promoting activity in barium sulfate-adsorbed serum resisted heating to 60°C. for 30 minutes, with some loss, but was destroyed at 70°C. in 10 minutes, coincident with gellation of the serum pro-

teins. Maximal precipitation of the active globulin fraction from diluted serum occurred over a wide range of acidity. No difference in activity was noted in fractions precipitated from pH 4.6 to 5.7. Neither adsorption with barium sulfate nor heating to 56°C. for 30 minutes induced the appearance of the clot-promoting fraction in the plasma in Case II.

With these data in mind, a crude preparation of the clot-promoting fraction was made by adsorbing pooled serum, which had been stored at 4°C. for 6 weeks, with powdered barium sulfate. The adsorbed serum was heated at 56°C. for 30 minutes, centrifuged, and the globulin fraction insoluble at pH 5.3 precipitated. Successive ammonium sulfate-insoluble fractions of this precipitate were then separated. In one such experiment, the greatest part of the clot-promoting activity was precipitated between 33 and 50 per cent saturation with ammonium sulfate, lesser amounts between 25 and 33 per cent, and little or no activity, between 0 and 25 per cent or above 50 per cent. In later studies, it seemed probable that the bulk of the activity was precipitated between 25 and 40 per cent saturation.

A number of tests were performed with this fraction. Even in high dilution it corrected the defect in the plasma in each case. For example, one part of clot-promoting fraction shortened the recalcified clotting time of 80 parts of Patient II's oxalated plasma from 45 minutes to 10 minutes.

The clot-promoting fraction was not thrombic, since it did not clot a solution of bovine fibrinogen. It did not alter the clotting time of a mixture of bovine thrombin and bovine fibrinogen. It prolonged slightly the clotting time of mixtures of bovine thrombin with either fresh plasma or plasma which had been refrigerated at 4°C. for 12 weeks.

The clot-promoting fraction did not possess appreciable thromboplastic or anti-hemophilic activity. One-sixth part of the fraction did not clot hemophilic platelet-deficient plasma in silicone-coated tubes. In Pyrex tubes, the fraction clotted hemophilic plasma in 150 minutes, but at this time partial clots were also seen in control tubes containing buffer instead of the fraction.

The fraction's lack of thromboplastic activity was also demonstrated by a study of the plasma of a patient with a circulating anticoagulant whose case was described in an earlier report (21). This patient's plasma clotted in a normal time upon the addition of highly diluted human brain thromboplastin. However, its anticoagulant effect was not overcome by a clot-promoting fraction prepared by heating barium sulfate-adsorbed fresh hemophilic plasma to 56°C. for 30 minutes and then precipitating the globulin fraction insoluble at pH 5.2.[8] Thus the fraction did not behave in the same way as tissue thromboplastin.

The clot-promoting fraction did not alter the one-stage prothrombin time of fresh normal plasma and contained no prothrombin, as determined by the method of Owren and Aas (9, 10). By a two-stage prothrombin method (11) the fraction did not influence the rate of evolution of thrombin by tissue thromboplastin in diluted plasma. Furthermore, it was without proaccelerin or proconvertin activity. Thus the fraction did not shorten the prolonged prothrombin time of normal plasma which had been stored at 4°C. for 2 months, and of plasma from a patient under treatment with Dicumarol®. This patient's prothrombin time was shortened by a one per cent solution of aged human serum, demonstrating that the plasma was deficient in proconvertin.

None the less, it was possible to demonstrate that the clot-promoting fraction had a profound effect on the conversion of prothrombin to thrombin in shed blood. One part of the fraction, in its original plasma concentration, was incubated for 1 hour at 37°C. with 8 parts of Patient I's whole blood. At the end of this time, the serum prothrombic activity was 4 per cent of plasma. In contrast, after one hour at 37°C. the serum prothrombic activity of this patient's blood alone was 43 per cent of plasma.

Similarly, the clot-promoting fraction decreased the serum prothrombic activity of *normal* blood strikingly. Thus, 20 minutes after 8 parts of blood were incubated at 37°C. with one part of buffer the serum prothrombic activity was 65 per cent of the prothrombic activity of plasma. On the other hand, 20 minutes after 8 parts of blood were mixed with one part of the fraction, the serum prothrombic activity was less than 10 per cent.

[8] This experiment was performed in February, 1950, in association with Dr. R. C. Hartmann and Dr. C. L. Conley.

Further incubation did not decrease the amount of prothrombin in the serum.

The clot-promoting activity in the crude fraction was only slightly diminished by heating at 60°C. for 30 minutes. The procedure outlined should not have removed all plasminogen activity. A clot formed from bovine fibrinogen and thrombin, in the presence of streptokinase and the fraction, lysed in 9 minutes; a control in which buffer was substituted for streptokinase did not lyse in 60 minutes.

The possible identity of the clot-promoting fraction with a previously described clotting factor

In a study of the relationship between the proteolytic activity of plasma and blood coagulation, a clot-accelerating property was observed in globulin preparations devoid of known clotting factors (1). These preparations and the clot-promoting fraction deficient in the plasma of the patients under study were made in a similar way. An attempt was made to determine whether these patients lacked the previously described factor.

Duplicating the previously published experiments, the globulin fraction of barium-sulfate adsorbed plasma, heated at 56°C. for 30 minutes, was prepared from normal plasma and from the plasma of Patient II. As had been reported previously, this globulin fraction of normal plasma accelerated the clotting of normal platelet-deficient plasma in silicone-lined tubes (Table VI). On the other hand, the globulin fraction prepared from the patient's plasma was virtually devoid of this activity. Furthermore, the more highly purified clot-promoting fraction of normal serum, which corrected the defect in the patient's plasma, also accelerated the clotting time of normal native plasma. Thus it seems likely that these patients are deficient in the clot-accelerating property described earlier.

TABLE VI

The effect of a globulin fraction prepared from heated, adsorbed, oxalated plasma upon the clotting time of normal native plasma

Concentration of globulin	Clotting time*	
	Normal globulin	Patient II's globulin
	min.	*min.*
Original plasma concentration	5	18
1/2 plasma concentration	6	20
1/4 plasma concentration	7	24
Buffer control	20	

* Clotting time in duplicate at 37°C. in silicone-coated tubes (internal diameter, 8 mm.) of mixtures of 0.4 ml. globulin solution and 0.2 ml. normal native plasma. The globulin solutions were prepared from oxalated plasma which had been adsorbed with barium sulfate and heated at 56°C. for 30 minutes. The globulin solutions were diluted with buffer.

The difference between the globulins of normal and abnormal plasmas could be demonstrated not only when the plasmas had been oxalated, adsorbed with barium sulfate, and heated, but also in globulin prepared in silicone tubes from native plasma. In repeated experiments, normal native plasma globulin greatly accelerated the clotting time of normal native plasma. The effect of two such globulins is demonstrated in Table VII. The globulin fraction of Patient II's native plasma, on the other hand, had virtually no clot-promoting effect.

This same experiment was performed, comparing hemophilic and normal plasmas. In both hemophiliacs tested, the clot-promoting effect was

TABLE VII

The effect of native globulin upon the clotting time of normal native plasma

Concentration of globulin	Clotting time (*min.*)*				
	Normal A	Normal B	Patient II	Normal C	Hemophiliac
Original plasma concentration	3	4	13	4	8
1/2 plasma concentration	4	6	17	5	9
1/4 plasma concentration	5	8	18	8	11
1/8 plasma concentration	5	11	18	10	13
Buffer control	17	17	17	34	34

* Clotting time of a mixture of 0.4 ml. globulin, prepared in silicone-coated tubes from native plasma, and 0.2 ml. normal native plasma. The globulin was diluted serially with buffer. Clotting times were measured in duplicate at 37°C. in silicone-coated tubes (internal diameter, 11 mm.). The normal native plasmas tested with normal globulins A and B and Patient II differed in each case, but normal Globulin C and the hemophilic globulin were tested simultaneously with the same native plasma.

less than that of normal globulin, although still much greater than that of plasma from Patient II (Table VII). Similar results were obtained testing the globulins prepared from the oxalated plasmas of a normal individual, a hemophiliac, and Patient III simultaneously.

DISCUSSION

The initiation of clotting in shed human blood has been unravelled in large measure through the study of individuals with one or another hemorrhagic disorder. As a result of such studies, a number of soluble components of plasma have been implicated in the evolution of thromboplastic activity in shed blood. These components include the anti-hemophilic factor (or thromboplastinogen) (2, 22–24), plasma thromboplastin component (or Christmas factor) (16–18), plasma thromboplastin antecedent (19), and a fourth plasma thromboplastin component recently described by Spaet, Aggeler, and Kinsell (20). In addition, there is evidence that prothrombin cannot be converted to thrombin optimally until the plasma or tissue thromboplastin reacts in some manner with proaccelerin (or Factor V, plasma ac-globulin or labile factor) (25) and proconvertin (or serum prothrombin conversion accelerator, Factor VII or co-thromboplastin) (25–28) to develop "effective" thromboplastic activity. Platelets may be needed for the development of maximal amounts of effective thromboplastic activity in plasma (23, 27–31), but probably not in systems containing tissue thromboplastin.

The three patients described in the present report seem to reveal hitherto unappreciated mechanisms involved in the development of plasma thromboplastic activity. These patients were remarkable in that they all had abnormally long clotting times without apparent ill effects. The long clotting time was the result of a defect in the plasma, since platelet-deficient plasma was incoagulable in glass. This defect was due neither to the presence of a circulating anticoagulant, nor to a deficiency in any of the following clotting factors, namely, fibrinogen, prothrombin, proaccelerin, proconvertin, anti-hemophilic factor, plasma thromboplastin component, plasma thromboplastin antecedent, the fourth thromboplastin component, staphylocoagulase-reacting factor, nor any factors needed for the action of trypsin on prothrombin (32) or for the conversion of fibrinogen to fibrin by thrombin (7). The normal thrombin and prothrombin times suggested that the defect was at an early stage in the clotting process, presumably in the plasma's intrinsic capacity to initiate the conversion of prothrombin to thrombin through some as yet unidentified mechanism. This was confirmed by the slightly increased serum prothrombic activity in Cases I and II, indicative of a defect in the conversion of prothrombin to thrombin.

The unique nature of the patients' abnormality was confirmed by the preparation of a globulin fraction of normal plasma or serum which corrected the defect. The active principle was separated from fibrinogen, anti-hemophilic globulin and proaccelerin by the use of serum instead of plasma, and from prothrombin, proconvertin and plasma thromboplastin component by adsorption of these substances upon barium sulfate. It resisted heating at 60°C. which is said to inactivate the fourth thromboplastin component (20). The method of preparation was such that one could not be sure that the fraction was devoid of plasma thromboplastin antecedent. However, this substance is said to be present in Cohn fractions III and IV of plasma, but not in fraction II (33). Preliminary studies suggest that both Cohn fractions II and III are effective in correcting the patients' defect.

Moreover, the normal globulin fraction seemed to be active at that stage of the clotting process defective in the abnormal plasmas. The fraction was neither thrombic nor thromboplastic. It did not alter the rate at which fibrinogen was converted to fibrin by thrombin, nor the rate at which prothrombin was converted to thrombin by tissue thromboplastin. However, the fraction tremendously accelerated the rate at which prothrombin was converted to thrombin when either normal or abnormal whole blood was allowed to clot in glass tubes. Thus the fraction was probably concerned with an early step of clotting, presumably the evolution of thromboplastic activity in shed blood.

Since the whole blood clotting time of each patient was prolonged, it is likely that the clot-promoting fraction is necessary for the optimal clotting of blood *in vitro*. The active globulin frac-

tion of normal plasma not only corrected the patients' defect, but accelerated the clotting of normal plasma as well. This suggests that the clot-promoting fraction may be present in the form of a precursor which is activated when blood is shed. The manner in which the fraction is activated by the precipitation of the globulin was not clarified. Since the globulin prepared in silicone tubes from normal native plasma was effective, exposure to wettable surfaces or the addition of oxalate was not necessary. The activation was not the result of the dilution which ordinarily takes place during preparation of the globulin, since an active globulin could be prepared by dialyzing serum against a buffer solution of low ionic strength (0.02) at pH 5.5, without any change in volume. Moreover, in incomplete experiments, a mixture of the precipitated globulin recombined with the supernatant dialyzed plasma still possessed clot-promoting activity. Both dilution and dialysis greatly reduce the concentration of many dialyzable components of the plasma, but simple dialysis of serum against barbital-saline buffer at pH 7.5 did not appear to activate the clot-promoting fraction.

The means by which the clot-promoting fraction influenced the development of thromboplastic activity were not defined. It was of interest that hemophilic native globulin was less effective than the normal in shortening the clotting time of normal native plasma, even though hemophilic plasma contained the same amount of the clot-promoting fraction as normal plasma. One hypothesis, then, is that the clot-promoting fraction exerts its action in association with the anti-hemophilic factor. This would be compatible with the observation that the clot-promoting fraction did not significantly alter the clotting time of native hemophilic plasma. Further experiments to test this hypothesis are in progress.

The bulk of the experiments reported refer to phenomena observed *in vitro*. It is noteworthy that the transfusion of normal plasma corrected the defect in Patient I. However, within 16 hours, his clotting time was once more abnormal. Thus in this patient the clot-promoting fraction seemed to have been utilized or destroyed rapidly.

The stability and solubility characteristics of the clot-promoting fraction are such that one would expect that its effect had been observed and recorded by other investigators. The fraction has many features in common with an accelerator of clotting, described in an earlier study, which seemed responsible for the clot-promoting effect of crude preparations of plasmin (1). Milstone (34, 35) has described a fraction of bovine globulin which had been adsorbed with barium sulfate. This fraction, when incubated with calcium ions, was activated to form a complex, named thrombokinase, which converted prothrombin to thrombin. Its method of preparation was such that it may well have been rich in the clot-promoting fraction described in the present paper. Tocantins, Carroll, and Holburn (36) observed that a euglobulin preparation of hemophilic plasma accelerated the clotting of normal blood. Although their interpretation differed, it is possible that these experiments can be explained by the presence of the clot-promoting fraction. Similarly, Nolf (37), Lenggenhager (38), Feissly (39), Travis and Ferguson (40) and others have described clot-enhancing preparations of plasma the activity of which may be partially explained by their content of the clot-promoting fraction.

The scientific literature dealing with problems of blood coagulation has been confused by a nomenclature which is complex and conflicting. It seems unwise to name the clot-promoting fraction described in the present report, or the trait manifest by its absence, until more is known of its mechanism of action. Temporarily one may designate the fraction by the name of Patient I, as Hageman factor, and its deficiency, as Hageman trait. The fraction is under continued, intensive study. Finally, it is noteworthy that it is possible to have a prolonged clotting time for many years without obvious harm, and in spite of operative procedures, childbirth, and menses. Presumably, the hemostatic mechanisms are such that they can protect these patients from injury. How these mechanisms operate to overcome the defect in coagulation is yet to be determined.

SUMMARY

1. A familial trait, occurring in both sexes, characterized by a prolonged clotting time without significant hemorrhagic symptoms, is described. The prolonged clotting time was apparently due to a defect in the production of plasma thromboplastic activity in shed blood.

However, the trait could not be identified with known hemorrhagic disorders.

2. A clot-promoting fraction apparently distinctive from known clotting factors could be prepared from normal barium sulfate-adsorbed, heated serum. This fraction not only corrected the defect in the patient's plasma but shortened the clotting time of normal platelet-deficient plasma. Data are presented relative to the significance of this clot-promoting fraction.

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THE CARDIOHEMODYNAMIC EFFECTS OF VENOUS CONGESTION OF THE LEGS OR OF PHLEBOTOMY IN PATIENTS WITH AND WITHOUT CONGESTIVE HEART FAILURE [1]

By WALTER E. JUDSON, WILLIAM HOLLANDER,[2] J. D. HATCHER,[3] MEYER H. HALPERIN, AND IRWIN H. FRIEDMAN [4]

(From the Robert Dawson Evans Memorial Department of Clinical Research and Preventive Medicine, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.)

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The purpose of this study was to clarify the cardiohemodynamic responses to acute reduction in the "effective circulating blood volume" in man. McMichael and Sharpey-Schafer found that the cardiac output in normal subjects fell during venous congestion of the extremities (1). They attributed this reduction in cardiac output to a decrease in the right atrial pressure. Their observation that right atrial pressure usually falls during such acute reductions in the "effective circulating blood volume" was confirmed by Warren, Brannon, Stead, and Merrill (2), but these latter workers did not usually find an associated decrease in cardiac output. They therefore suggested that the cardiac output response is independent of changes in right atrial pressure, at least within physiological limits. However, observations by Howarth, McMichael, and Sharpey-Schafer (3) on the effects of phlebotomy and of venous congestion of the extremities in patients with congestive heart failure supported the concept that the level of right atrial pressure is an important factor in determining cardiac output. The studies reported herewith differ in certain aspects from all those just mentioned.

MATERIAL AND METHODS

The patients in Group I had neither clinical nor hemodynamic evidence of cardiac insufficiency at rest or after exercise. They included two normal subjects, one with essential hypertension, and two with hypertensive cardiovascular disease. Group II-A contained patients with non-valvular types of heart disease with congestive failure and included three patients with hypertensive cardiovascular disease and two with arteriosclerotic heart disease. Group II-B contained patients with valvular types of heart disease and congestive failure and included five with rheumatic heart disease and one with syphilitic heart disease. All of the patients in Group II-A and B were considered to be in congestive failure on the basis of their clinical cardiovascular symptoms and signs and their abnormal hemodynamic responses during exercise. Furthermore, all of the patients were taking digitalis, and several were receiving mercurial or other diuretics for treatment of their congestive failure. Group III consisted of patients with pulmonary emphysema, two of whom had cor pulmonale with congestive heart failure and two of whom had pulmonary hypertension with no evidence of right ventricular hypertrophy or failure.

PROCEDURE

The patients were studied in the post-absorptive state without any preliminary sedative medication. A double-lumen intracardiac catheter was inserted into the right heart so that simultaneous records of the pulmonary arterial and right ventricular pressures could be obtained. An indwelling needle was maintained in the brachial artery. All pressures were measured with electromanometers [5] and recorded by direct-writing oscillograph; the zero point of reference for all pressures was 10 cm. above the back of the supine subject. Mean pressures were determined by electrical integration. Using criteria which are comparable with those in Cournand's laboratory, reductions in either the right ventricular diastolic or mean pulmonary arterial pressures of 5 mm. Hg or more were considered physiologically significant, while changes in the mean systemic arterial pressure of 15 mm. Hg were also judged physiologically significant (4).

The resistances were calculated, according to Gorlin and his co-workers (5), by the following formulae:

The "total pulmonary" resistance (actually, total

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[2] United States Public Health Service Fellow in Medicine, Evans Memorial, Massachusetts Memorial Hospitals.

[3] Formerly Fellow in Medicine, Evans Memorial, Massachusetts Memorial Hospitals. Present Address: Queen's University, Kingston, Ontario, Canada.

[4] Formerly United States Public Health Service Fellow in Medicine, Evans Memorial, Massachusetts Memorial Hospitals. Present Address: East Meadow, New York.

[5] Sanborn Co., Cambridge, Massachusetts.

TABLE I

Individual data *

Patient Age Sex Surface area (*sq. m.*)	Clinical classification	Procedure	Duration *Minutes*	Cardiac output *L./min.*	Oxygen consumption *Ml./min.*	A-V oxygen difference *Vol. %*	Arterial oxygen saturation %	Hematocrit (arterial) %	Pressure (*mm. Hg*)							Pulse rate *Beats/min.*	Resistances *Dynes seconds cm.*$^{-5}$	
									Pulmonary artery			Right ventricular end-diastolic	Systemic arterial				Total systemic	"Total pulmonary"
									S	D	M		S	D	M			
Group I—Normal or compensated heart disease																		
W. Y. 36 M 1.68	Normal	Control	—	7.57	249	3.29	95	42	24	12	16	—	120	80	100	60	1,060	170
		Congestion	7	5.98	224	3.75	95	42	23	12	16	—	110	80	95	62	1,270	210
			27	5.96	231	3.88	94	41	24	12	16	—	110	80	95	62	1,270	210
L. A. 47 F 1.53	Normal	Control	—	6.71	247	3.68	93	39	—	—	—	—	—	—	—	—	—	—
		Congestion	13	6.12	257	4.20	88	40	—	—	—	—	—	—	—	—	—	—
W. D. 36 M 2.00	H.C.V.D.	Control	—	8.12	319	3.93	95	44	25	9	14	—	210	125	155	73	1,530	140
			—	7.48	314	4.20	94	44	25	9	14	—	210	125	155	78	1,660	150
		Congestion	20	6.70	307	4.58	96	43	15	6	9	—	167	116	135	70	1,610	110
M. R. 44 F 1.50	Ess. hypert.	Control	—	7.79	236	3.03	94	38	24	13	15	5	180	90	120	112	1,230	150
		Congestion	55	5.22	225	4.31	95	37	22	13	14	5	160	100	120	112	1,840	210
I. R. 42 M 1.96	H.C.V.D.	Control	—	9.67	357	3.69	94	44	32	14	20	3	210	100	145	72	1,200	160
				9.95	382	3.84	93	44	32	14	20	3	210	100	145	72	1,160	160
		Congestion	49	8.04	336	4.18	95	43	32	14	20	3	200	100	140	70	1,390	200
Group II-A—Decompensated (non-valvular) heart disease																		
M. G. 52 F 1.70	H.C.V.D. N.S.R. III C	Control	—	5.07	409	8.06	89	44	70	45	55	10	250	150	200	84	3,150	870
		Congestion	21	5.51	388	7.03	87	44	60	30	42	5	250	150	200	76	2,900	610
			47	5.32	371	6.98	87	45	55	30	40	5	250	150	200	76	3,000	600
J. D. 38 M 1.65	H.C.V.D. N.S.R. IV E	Control	—	2.38	290	12.17	95	44	70	50	58	30	220	160	180	108	6,040	1,950
		Congestion	50	2.46	261	10.63	94	44	70	50	58	25	220	160	180	108	5,850	1,880
D. C. 48 M 1.91	H.C.V.D. N.S.R. III C	Control	—	9.16	449	4.90	92	44	65	32	42	15	210	90	140	86	1,220	370
		Phlebotomy (450 ml.)	40 +10†	10.26	472	4.60	93	43	55	31	39	9	210	85	140	90	1,090	300
F. A. 61 F 1.85	A.S.H.D. N.S.R. III D	Control	—	4.09	229	5.60	94	55	62	40	47	18	118	66	84	92	1,660	920
			—	3.71	221	5.95	100	55	62	40	47	18	120	70	85	84	1,830	1,010
		Phlebotomy (500 ml.)	26 + 8	4.13	240	5.81	98	53	60	40	45	17	110	70	80	92	1,550	870
M. C. 47 F 1.69	A.S.H.D. A.F. III C	Control	—	7.64	343	4.49	93	37	68	32	45	20	185	70	115	112	1,200	470
			—	7.95	349	4.39	91	36	68	32	45	20	185	70	115	112	1,160	450
		Congestion	31	8.08	360	4.46	93	36	68	32	45	20	185	70	115	108	1,140	440
			68	7.73	337	4.36	95	35	68	32	45	20	185	70	115	110	1,190	460
		Recovery	30	8.15	360	4.42	92	37	68	32	45	20	185	70	115	108	1,130	440

* Symbols used in table—A.S.H.D., arteriosclerotic heart disease; H.C.V.D., hypertensive cardiovascular disease; R.H.D., rheumatic heart disease; Syph.H.D., syphilitic heart disease; Pul. emph., pulmonary emphysema; M.I., mitral insufficiency; M.S., mitral stenosis; A.I., aortic insufficiency; A.S., aortic stenosis; T. I., tricuspid insufficiency; N.S.R., normal sinus rhythm; A.F., auricular fibrillation; S, systolic; D, diastolic; M, mean.

† First figure is duration of phlebotomy, and second is interval from end of phlebotomy to start of cardiac output determination.

TABLE I—*Continued*

Patient Age Sex Surface area (*sq. m.*)	Clinical classification	Procedure	Duration *Minutes*	Cardiac output *L./min.*	Oxygen consumption *Ml./min.*	A-V oxygen difference *Vol. %*	Arterial oxygen saturation %	Hematocrit (arterial) %	Pressure (*mm. Hg*)							Pulse rate *Beats/min.*	Resistances *Dynes seconds cm.*$^{-5}$	
									Pulmonary artery			Right ventricular end-diastolic	Systemic arterial				Total systemic	"Total pulmonary"
									S	D	M		S	D	M			
Group II-B—Decompensated (valvular) heart disease																		
L. G. 36 F 1.54	R.H.D. A.S. N.S.R. III C	Control	—	3.04	248	8.17	95	36	98	48	65	27	95	65	75	104	1,970	1,710
			—	3.02	255	8.44	98	36	98	48	65	27	95	65	75	104	1,980	1,720
		Congestion	42	3.21	240	7.49	98	35	90	43	60	18	95	65	75	96	1,870	1,490
W. T. 40 M 1.89	R.H.D. A.S. M.I. & M.S. A.F. III D	Control	—	4.62	301	6.51	94	40	68	36	50	19	140	80	100	50	1,730	860
			—	4.33	306	7.06	95	40	68	36	50	19	140	80	100	50	1,850	920
		Congestion	53	4.97	304	6.12	90	40	60	33	43	12	125	80	90	52	1,450	690
H. A. 50 M 1.74	Syph. H.D. A.I. N.S.R. III B	Control	—	5.81	284	4.89	92	37	48	16	25	4	255	105	160	84	2,200	340
		Congestion	26	5.42	262	4.84	92	36	22	10	15	0	195	95	125	76	1,840	220
R. O. 40 F 1.40	H.C.V.D. R.H.D. M.S. & M.I. A.F. IV E	Control	—	4.43	219	5.05	99	36	78	39	53	10	220	115	165	86	2,980	960
		Congestion	43	4.24	205	4.84	96	37	60	27	40	5	210	115	160	84	3,020	750
M. W. 50 F 1.35	R.H.D. M.S. & M.I. & T.I. A.F. IV E	Control	—	1.56	144	12.41	88	50	—	—	—	35/25(28)‡	140	90	110	80	5,640	—
		Phlebotomy (700 ml.)	40 +14	2.39	158	8.91	86	45	—	—	—	20/10(13)	120	70	80	80	2,680	—
J. Di. 37 M 1.68	R.H.D. M.I. A.F. IV E	Control	—	2.76	226	8.18	93	38	35	17	23	10	110	60	75	42	2,170	670
			—	2.98	225	7.55	93	38	35	17	23	10	110	60	75	42	2,010	620
		Congestion	30	3.13	221	7.07	93	38	35	14	22	—	100	60	70	48	1,790	560
Group III—Chronic pulmonary disease																		
D. K. 49 M 1.86	Pul. emph. N.S.R. II B	Control	—	7.03	320	4.55	91	44	40	24	28	5	160	100	125	82	1,420	320
		Congestion	28	6.85	317	4.63	91	44	40	24	28	5	150	90	120	86	1,400	330
C. Di. 63 M 1.85	Pul. emph. Cor pulmonale N.S.R. II B	Control	—	6.27	259	4.13	90	56	45	27	32	5	140	70	100	60	1,280	410
		Phlebotomy (600 ml.)	10 +19	5.52	233	4.22	92	55	40	25	28	1	130	65	95	62	1,380	410
W. H. 56 M 2.00	Pul. emph. Cor pulmonale in failure N.S.R. IV E	Control	—	6.97	308	4.42	75	71	70	50	60	20	125	80	105	88	1,200	690
		Congestion	59	5.73	322	5.62	77	72	60	45	53	20‡	125	80	105	88	1,460	740
		Phlebotomy (750 ml.)	34 +12	6.13	334	5.43	79	71	60	40	50	20	110	60	85	90	1,110	650
W. Da. 66 M 1.76	Silicosis Cor pulmonale in failure N.S.R. III D	Control	—	6.51	244	3.75	52	50	82	45	60	25	130	70	100	80	1,230	740
		Phlebotomy (600 ml.)	10 + 3	4.88	249	5.10	54	47	82	45	60	25	130	70	100	80	1,640	980

‡ Right Atrial Pressure.

resistance opposing the right ventricle) was obtained from the following equation:

$$R = \frac{PA_m}{CO} \times 1{,}332 \text{ dynes seconds cm.}^{-5}$$

The "total systemic peripheral" resistance (actually, total resistance opposing the left ventricle) was obtained similarly:

$$R = \frac{BA_m}{CO} \times 1{,}332 \text{ dynes seconds cm.}^{-5}$$

where PA_m = pulmonary arterial mean pressure, mm. Hg
BA_m = brachial arterial mean pressure, mm. Hg
CO = cardiac output, ml. per second
1332 = conversion factor from mm. Hg to dynes per cm.^{5}

After preliminary practice, one or two control cardiac outputs were determined by the direct Fick method. Expired air was collected for two minutes in a Douglas bag while simultaneous blood samples were obtained at a constant rate from the brachial and pulmonary arteries. Immediately before and after the Fick procedure, pressures in the pulmonary artery, right ventricle, and brachial artery were determined. The volume of inspired air was measured by a Tissot spirometer, which contained a supply of outdoor air. Oxygen and carbon dioxide concentrations in the expired air were determined by the Haldane method (6) with duplicated determinations required to agree within 0.03 per cent. Blood samples were analyzed for oxygen content, capacity, and saturation by the method of Van Slyke and Neill (7) with duplicate determinations required to agree within 0.10 vol. per cent. Possible slight errors in blood oxygen contents due to dilution in obtaining blood samples through the catheter were corrected as follows:

Corrected venous oxygen content

$$= \text{Observed venous oxygen content} \times \frac{\text{art. hct.}}{\text{ven. hct.}}$$

In fourteen patients the cardiohemodynamic measurements were repeated 7 to 68 minutes after application of venous congesting cuffs high on the thighs at 70 mm. Hg. In six patients similar measurements were made from 3 to 20 minutes after the completion of a 450 to 750 milliliter phlebotomy. The venesections were accomplished over a period of 10 to 40 minutes. A few patients in whom the oxygen consumption during the control and procedure periods did not check within 10 per cent were excluded from the study. Likewise, patients in whom the cardiac output during the control periods varied more than 10 per cent were also excluded from the study (4).

RESULTS

The cardiovascular responses to venous congestion of the extremities and phlebotomy are summarized in Table I and the means are shown in Table II with a statistical analysis.

TABLE II

Mean data and statistical analysis

		Cardiac output *L./min.*	Oxygen consumption *ml./min.*	A-V oxygen difference *Vol. %*	Arterial oxygen saturation %	Hematocrit (arterial) %	Pulmonary artery			Right ventricular end diastolic *mm. Hg*	Systemic arterial			Pulse rate *Per min.*	Resistances *Dynes seconds cm.*$^{-5}$	
							Systolic *mm. Hg*	Diastolic *mm. Hg*	Mean *mm. Hg*		Systolic *mm. Hg*	Diastolic *mm. Hg*	Mean *mm. Hg*		Total systemic	Total pulmonary artery
Group I (Compensated) n = 5	Control	*7.94 ±0.49**	*284 ±25*	*3.56 ±0.20*	*93.6 ±0.5*	*41.4 ±1.2*	*26 ±3*	*12 ±1*	*16 ±2*		*180 ±21*	*99 ±10*	*130 ±12*	*80 ±11*	*1,270 ±110*	*150 ± 10*
	Congestion	*6.40 ±0.51*	*271 ±21*	*4.22 ±0.10*	*93.8 ±1.5*	*40.8 ±1.2*	*23 ±4*	*11 ±2*	*15 ±2*		*159 ±19*	*99 ± 7*	*122 ±12*	*78 ±11*	*1,530 ±120*	*180 ± 30*
	Difference	*−1.53 ±0.33*	*−13 ± 7*	*+0.66 ±0.16*	*+0.2 ±1.1*	*−0.6 ±0.4*	*−3 ±2*	*−1 ±1*	*−1 ±1*		*−21 ± 8*	*0 ± 4*	*−8 ± 4*	*−2 ± 1*	*+260 ±120*	*+30 ± 20*
	Significance of difference (P)	<0.01	0.15	0.02	0.87	0.20	0.30	0.39	0.30		0.08	>0.9	0.18	0.21	0.13	0.25
Group II-A & B (Decompensated) n = 11	Control	*4.59 ±0.69*	*286 ±27*	*7.33 ±0.85*	*93.4 ±0.6*	*41.8 ±1.9*	*66 ±6*	*36 ±4*	*46 ±4*		*177 ±18*	*96 ±10*	*128 ±13*	*84 ± 7*	*2,730 ±500*	*920 ±170*
	Congestion	*4.87 ±0.72*	*281 ±27*	*6.52 ±0.59*	*92.7 ±1.4*	*41.0 ±1.6*	*58 ±6*	*31 ±4*	*41 ±4*		*165 ±17*	*93 ±10*	*120 ±14*	*83 ± 6*	*2,300 ±410*	*780 ±160*
	Difference	*+0.28 ±0.13*	*−5 ± 5*	*−0.81 ±0.36*	*−0.7 ±0.6*	*−0.8 ±0.5*	*−9 ±3*	*−5 ±2*	*−5 ±2*	*−6 ±1*	*−12 ± 5*	*−3 ± 2*	*−8 ± 4*	*−1 ± 2*	*−430 ±810*	*−140 ± 30*
	Significance of difference (P)	0.05	0.36	0.02	0.32	0.12	0.01	0.03	<0.01	<0.01	0.06	0.17	0.06	>0.90	0.60	0.01
Difference between response of Groups I & II	Difference	*−1.81 ±0.35*	*−8 ± 9*	*+1.47 ±0.35*	*+0.9 ±1.3*	*+0.2 ±0.6*	*+5 ±4*	*+6 ±2*	*+4 ±2*		*− 9 ± 9*	*+3 ± 4*	*+0.6 ±6*	*−1 ± 2*	*+690 ±900*	*+170 ± 30*
	Significance of difference	0.01	0.40	<0.01	0.50	0.73	0.17	0.06	0.08		0.33	0.46	>0.90	0.86	0.46	<0.01

* Figures in italics are standard error.

Group I—Patients without heart failure

A. Cardiac output and oxygen measurements. All five patients showed falls in cardiac output, four of which were greater than 10 per cent. The mean decrease in cardiac output was 1.53 liters per minute which was highly significant statistically. This was associated with a mean increase of 0.66 vol. per cent in the arterio-venous oxygen difference, a change which was also highly significant. The oxygen consumption, arterial oxygen saturation, hematocrit, and pulse rate did not change significantly.

B. Vascular pressures. In two patients (M. R., I. R.) in whom it was measured, the end-diastolic right ventricular pressure did not change measurably from the control normal values during congestion of the limbs despite a decrease in the cardiac output. Only one patient (W. D.) showed a physiologically significant decrease in the pulmonary arterial pressure. The group as a whole showed no significant change in the pulmonary arterial pressure. There was a tendency for the peripheral arterial pressure to decrease, but the change was not statistically significant for the group. Indeed, only one severely hypertensive patient, W. D., had an appreciable drop in blood pressure.

C. Vascular resistance. Both the "total pulmonary" and "total systemic" resistances showed slight but not significant increases. The direction of these slight changes was such as to tend to maintain constancy of pressure with the change in cardiac output. In only one patient, W. D., who had a considerable fall in arterial pressure did a correspondingly large change in the systemic vascular resistance occur.

Group II—Patients with heart failure

The decompensated patients with valvular and non-valvular heart disease responded similarly to stimuli which reduced the "effective blood volume." The averages presented in Table II include both types of patients.

A. Cardiac output and oxygen measurements. On the average there was a slight but statistically significant increase in cardiac output of 0.28 liters per minute. This change was associated with a mean decrease of 0.81 vol. per cent of the arterio-venous oxygen difference, which was also significant statistically. The direction of these responses was opposite to that of the compensated patients, and the difference between the responses of the two groups was highly significant statistically. The oxygen consumption, arterial oxygen saturation, hematocrit, and pulse rate did not change significantly.

B. Vascular pressures. Initially the right ventricular end-diastolic pressures were elevated in almost all the patients. Venous congestion of the extremities or venesection produced a mean decrease of 6 mm. Hg in the right ventricular diastolic pressure in the group, a highly significant change statistically and physiologically. The pulmonary arterial systolic, diastolic, and mean pressures likewise showed significant decreases of a similar order of magnitude. The pulmonary arterial pressures in these patients changed in the same direction as in the compensated group but to a significantly greater degree. On the average there was an 8 mm. Hg decrease in peripheral mean arterial pressure, a change which was not statistically significant.

An interesting illustration is M. W., a patient with mitral valvular heart disease and functional tricuspid insufficiency. This patient had the largest increase in cardiac output of the entire group after venesection, and this was associated with the greatest reduction in mean right atrial pressure. After phlebotomy the mean pressure and the amplitude of pulsations in the right atrial pressure tracing, as observed in Figure 1, were reduced, possibly due to a decrease in the volume of blood regurgitated through the tricuspid valve. The cardiac output had increased 53 per cent.

However, not all the patients in the group who had increases in the cardiac output during these maneuvers had decreases in the vascular pressures. Conversely, not all the patients who had significant reductions in the right ventricular diastolic and the pulmonary arterial pressures had significant increases in cardiac output. Furthermore, in those patients in whom there were simultaneously measured increases in cardiac output there was no consistent correlation with the degree of reduction in the right ventricular diastolic and pulmonary arterial pressures.

C. Vascular resistances. The "total pulmonary" resistance decreased, on the average, 14 per cent, a highly significant statistical change for the

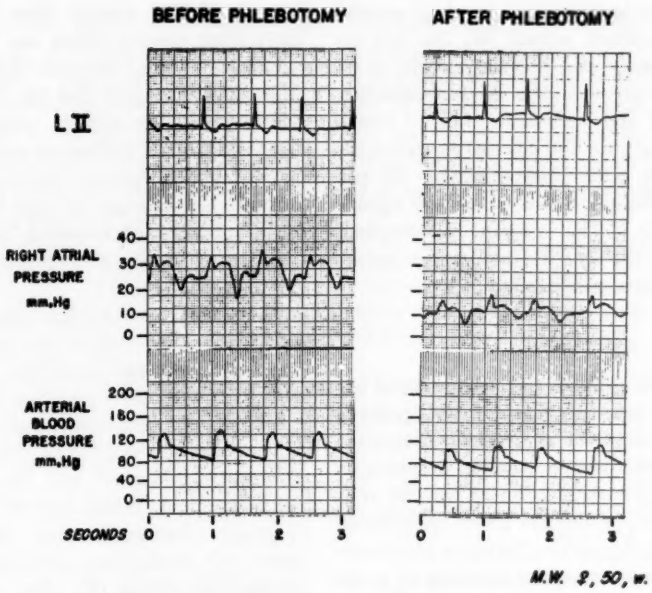

FIG. 1. RECORDING OF THE RIGHT ATRIAL PRESSURE IN PATIENT (M. W.) WITH RHEUMATIC HEART DISEASE AND FUNCTIONAL TRICUSPID INSUFFICIENCY SHOWING THE REDUCTION IN MEAN PRESSURE AND AMPLITUDE OF PULSATIONS AFTER PHLEBOTOMY

This change may be due to reduction in degree of regurgitation. This patient likewise had a marked increase in cardiac output.

group. The total systemic resistance also decreased 16 per cent, but the change lacked statistical significance. The vascular resistances changed in a direction such that the blood pressure tended to remain at a constant level with the simultaneous increase in cardiac output. The decreases in resistances in this group contrasted with the increases in the compensated patients. The difference between the responses of the "total pulmonary" resistance of the two groups was highly significant statistically.

Group III—Patients with pulmonary emphysema with or without heart failure

The responses to venesection of two patients with heart failure due to pulmonary heart disease were different from those with heart failure due to forms of heart disease generally classified as "low output failure." Therefore, these patients as well as two others with actual or potential cor pulmonale without heart failure are presented separately from the other groups. The small number of patients studied rendered statistical analysis of the results impractical.

The two patients (D. K., C. Di.) in this group without heart failure had elevated pulmonary arterial pressure, distinguishing them from the patients in Group I. Both of these patients had reductions in cardiac output during limb congestion (D. K.) or after phlebotomy (C. Di.). However, in patient C. Di., the decrease in cardiac output was proportionate to a fall in the oxygen consumption without an appreciable change in the mixed arterio-venous oxygen difference. In this patient there was a fall in the right ventricular end-diastolic and pulmonary arterial pressures, while in patient D. K., there were no changes in these pressures.

The two patients (W. H., W. Da.) in this group with congestive failure had appreciable decreases in cardiac output after phlebotomy with no changes in the right ventricular end-diastolic pressure. Patient W. H., showed a moderate

decrease in the pulmonary and brachial arterial pressures. In contrast, patient W. Da. had no fall in the pulmonary and brachial arterial pressures even in the presence of a marked reduction in cardiac output and hematocrit. There was a very slight tendency for the arterial oxygen saturation to increase after phlebotomy (+ 1.7 per cent), but the change lacked statistical significance ($P = 0.10$). The oxygen consumption, hematocrit, and pulse rate did not change appreciably in the group as a whole.

DISCUSSION

Normal subjects, patients with compensated hypertensive cardiovascular disease, and patients with cor pulmonale with or without congestive failure all appeared to have a similar response in cardiac output after venesection or venous congestion of the limbs, procedures which are believed to produce a reduction in the "effective blood volume." This response was characterized by a decrease in cardiac output which was usually not associated with measurable changes in the right ventricular end-diastolic pressure. In contrast, patients with congestive heart failure due to hypertension, coronary, or valvular heart disease had usually a slight increase, occasionally no change, or rarely a slight decrease in cardiac output. Again these changes in cardiac output were observed in individual cases to be independent of changes in the right ventricular end-diastolic pressure, which either remained the same or decreased. The group as a whole, however, showed a significant reduction in the right ventricular diastolic pressure.

Collateral studies in this laboratory have shown that patients with different types of congestive failure also may show characteristic changes in cardiac output in response either to exercise (6) or to the acute infusion of saline solution (7). Thus, in response to these stimuli, normal subjects, patients with compensated heart disease, and patients with cor pulmonale in failure usually increase their cardiac outputs. Patients with hypertension, coronary artery, and valvular heart disease with congestive failure have relatively fixed cardiac outputs which do not *increase* normally after exercise or saline infusion. Furthermore, as shown in the present study, the cardiac output in such patients usually does not *decrease* normally after stimuli which are believed to cause reductions in the "effective blood volume." The observations suggest that the heart in hypertensive heart disease without congestive failure and in cor pulmonale without or even with congestive failure may have greater reserve and may be *more* responsive to changes in right heart filling, while in the patients with so-called "low output failure" the heart is usually *less* responsive to changes in right heart filling.

The responses in cardiac output in the present study are similar to those previously reported (1). However, increases in cardiac output in patients with "low output" congestive heart failure were observed less frequently, and when observed, were smaller in magnitude than those found by Howarth, McMichael, and Sharpey-Schafer (3). An increase in cardiac output has been reported to occur occasionally after phlebotomy in patients with chronic cor pulmonale and severe congestive heart failure (8). By contrast, in our two cases of chronic pulmonary disease with severe congestive failure the cardiac output fell after phlebotomy. As just mentioned, in a larger series of patients with cor pulmonale and congestive failure, a normal increase in cardiac output was frequently found in response to exercise or to an acute infusion of hypertonic saline solution (9).

Warren, Brannon, Stead, and Merrill (2) found that the response of cardiac output depends not solely upon changes in the right atrial pressure. McMichael, and his co-workers (1, 3, 10) have also observed falls in the right atrial pressure after reduction in "effective blood volume." Measurements of mean right atrial pressure, however, may not accurately reflect right ventricular filling pressure. The measurements of the right ventricular end-diastolic pressure obtained in the present study did not show a uniform decrease after venesection or trapping of blood in the extremities by venous congestion in patients with "low output" type of failure. Furthermore, the response in cardiac output was found to be independent of the changes in the right ventricular end-diastolic pressure. These results, however, do not necessarily bear upon the relevancy of Starling's Law to the intact human heart since neither the right ventricular end-diastolic volume nor the pericardial pressure was measured. The

data suggest, however, that factors other than right heart filling are also important in determining cardiac output. These factors might, and undoubtedly do, include some or all of the following: 1) the degree or "stage" of congestive failure and the associated state of myocardial metabolism. This factor would include those considerations ordinarily implied by the terms "myocardial reserve," "cardiac fatigue," and "cardiac tone"; 2) the involvement in the failure of one or more chambers of the heart. Thus, failure of the left and right ventricles might well behave differently than failure of either one alone; 3) the amount of pulmonary vascular and even of peripheral vascular disturbances associated with the failure; 4) the amount of mechanical defect such as valvular incompetence associated with the failure.

The main factors controlling pulmonary arterial pressure (in relation to intrapleural pressure) are cardiac output, pulmonary arteriolar resistance, and resistance to flow through the left side of the heart. Since the latter two measurements were not obtained in this study they cannot be evaluated. However, it can be stated that the reductions in cardiac output observed in normal subjects and in patients with cor pulmonale were not consistently associated with falls in pulmonary arterial pressure. In patients with left ventricular failure who had rises in cardiac output after phlebotomy the reduction in pulmonary arterial pressure was attributed partly to a decrease in resistance to flow because of a lower left atrial pressure. However, since "wedge" or "pulmonary capillary" pressures were not taken because of the already great complexity of the procedure, no definite observations bearing on this point are available.

CONCLUSIONS

During venesection or venous congestion of the limbs (acute reductions of "effective blood volume"),

1. Normal subjects, patients with compensated cardiovascular disease, and patients with cor pulmonale with or without failure had a fall in cardiac output.

2. Patients with congestive heart failure due to valvular, hypertensive, or coronary heart disease usually had a slight rise, occasionally no change, or rarely a slight fall in cardiac output.

3. The response in cardiac output was not necessarily accompanied by a change in the right ventricular end-diastolic pressure.

4. A reduction in the pulmonary arterial pressure was not consistently observed. Decreases in cardiac output in some patients may explain the fall in pulmonary arterial pressure.

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THE CEREBRAL AND PERIPHERAL UPTAKE OF AMMONIA IN LIVER DISEASE WITH AN HYPOTHESIS FOR THE MECHANISM OF HEPATIC COMA [1, 2]

By SAMUEL P. BESSMAN [3] AND ALICE N. BESSMAN [4]

(From the Research Foundation of Children's Hospital, Washington, D. C.)

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Hepatic coma is a frequent terminal event in cirrhosis of the liver. Interest in its pathogenesis and treatment has been stimulated by recent observations that there is some relation between the increased blood ammonia level and the development of coma (2–4).

In the course of a series of observations of the cerebral blood flow and oxygen uptake in a number of diseases, the cerebral arteriovenous difference of ammonia was also measured to test a hypothesis for the chemical mechanism of hepatic coma. This report deals with the cerebral and peripheral arteriovenous differences of ammonia in patients with cirrhosis of the liver, and in several other conditions.

METHODS

Arterial blood was obtained by femoral artery puncture and cerebral venous blood by cannulation of the jugular bulb by standard methods. Peripheral venous blood was

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[2] Presented in part at the Federation of Biological Scientists, Atlantic City, New Jersey, May, 1954 (1).

[3] Present Address: Dept. of Pediatrics, University of Maryland Medical School, Baltimore 1, Md.

[4] Fellow in Medicine, George Washington University Medical School.

TABLE I-A

Patients without hepatic disease, blood ammonia, gamma per ml.

Patient	Age	Diagnosis	Mental state	Arterial	Cerebral venous	Peripheral venous	Cerebral (A-V)	Peripheral (A-V)
V. P.	20	Post barbit. intox.	Alert, Oriented	0.80	0.72		.08	
R. G.	23	Post-anoxic brain damage	Semi-comatose	0.60	0.78		−.18	
				0.86	0.90		−.04	
I. C.	88	Hypertensive encephalopathy	Semi-comatose	0.33	0.31		.02	
C. W.	75	Hypoglycemic coma	Comatose	0.70	0.70		.00	
F. R.	53	Asthma	Alert	0.75	0.73		.02	
W. J.	73	Uremia	Semi-comatose	0.80	0.86		−.06	
W. J.	77	Arteriosclerosis, Hypertension	Senile	0.84	0.82		.02	
M. M.	66	Arteriosclerosis, Hypertension	Senile	0.79	0.72		.07	
A. G.	73	Parkinsonism	Senile	0.52	0.62		−.10	
M. W.	38	Cerebral aneurysm	Alert	0.92	0.97		−.05	
D. D.	40	Subdural hematoma	Comatose	0.80	0.88		−.08	
E. D.	47	Uremia, Hypertension	Lethargic	0.80	0.80		00	
F. C.	39	Barbit. intox.	Sleepy	0.77	0.82		−.05	
			Alert	0.98	1.13		−.15	
E. D.	47	Uremia	Comatose	0.70	0.66		.04	
F. M.	50	Post barbit. intox.	Alert, Oriented	0.88	0.76		.12	
		Mean		0.76	0.78		−.02	

TABLE I-B

W. C.	40	Post-op. ca. lung	Alert	0.73		0.97		−.23
L. A.	57	Diabetes	Alert	0.98		0.98		00
V. G.	56	Uremia	Semi-comatose	0.77		0.88		−.11
R. B.	60	Uremia	Comatose	0.82		0.92		−.10
J. W.	40	Alcoholism	Alert	0.98		0.90		.08
J. T.	72	Arteriosclerosis	Senile	0.79		0.86		−.07
		Mean		0.85		0.92		−.07
23 Observations on 21 Patients		Mean for all patients		0.78				

TABLE II-A

Patients with hepatic disease, blood ammonia in gamma per ml.

Patient	Age	Diagnosis	Mental state	Arterial	Cerebral venous	Peripheral venous	Cerebral (A-V)	Peripheral (A-V)
T. B.	51	Cirrhosis	Lethargic, Confused	3.90	3.58		0.32	
			Semi-comatose	3.94	3.25		0.69	
			Comatose	8.44	7.12		1.32	
A. J.	66	Cirrhosis	Slightly confused	2.25	2.05		0.20	
			Semi-comatose	4.70	4.46		0.24	
H. A.	59	Liver dis.	Confused 48 hrs. ago Alert at time of study	1.73	1.60		0.12	
W. S.	59	Cirrhosis	Acute coma	6.64	4.24		2.40	
L. P.	59	Cirrhosis	Confused	3.68	3.15		0.53	
			Semi-comatose	3.00	2.32		0.68	
V. W.	65	Liver dis. (Etiol. ?)	Comatose	2.81	2.50		0.31	
L. G.	51	Cirrhosis	Slightly confused	1.59	1.42		0.71	
J. D.	43	Cirrhosis	Confused	1.80	1.43		0.37	
W. M.	60	Cirrhosis	Lethargic, Confused	3.09	2.94		0.15	
L. M.	65	Carcinoma of liver	Confused	1.89	1.58		0.31	
L. H.	39	Cirrhosis and uremia	Comatose	3.19	2.69		0.50	
E. B.	44	Cirrhosis (Carcinoma?)	Comatose	2.75	2.56		0.19	
R. L.	49	Cirrhosis	Semi-comatose	2.83	2.49		0.34	
		Mean of Table II-A		3.43	2.90		0.52	

TABLE II-B

L. H.	39	Cirrhosis and uremia	Comatose	3.19		2.77		0.42
E. B.	44	Cirrhosis (Carcinoma?)	Comatose	2.75		2.25		0.50
R. L.	49	Cirrhosis	Semi-comatose	2.83		2.62		0.21
C. F.	47	Cirrhosis	Semi-comatose	2.16		1.71		0.45
A. S.	53	Liver dis. T. B. etiol.	Alert	1.39		1.27		0.12
W. C.	39	Portal-caval shunt, Cirrhosis	Lethargic, Oriented	1.77		1.15		0.62
R. W.	38	Cirrhosis	Lethargic, Oriented	2.17		1.42		0.75
		Mean of Table II-B		2.32		1.88		0.44
		Mean of the three patients with all three determinations		2.92	2.58	2.55	0.34	0.37

drawn from the antecubital fossa. Ammonia determinations were made by the method of Seligson and Seligson (5) modified in the following manner to permit measurement of smaller amounts of ammonia. The diffusion vessels (15 ml. penicillin vial stoppered with the standard diaphragm stopper through which was passed a 3 mm. in diameter glass rod enlarged at the tip to a 6 mm. ball) were loaded with 1.0 ml. of saturated potassium carbonate. The rods were dipped into normal sulphuric acid and replaced in the vials. Blood was taken without stasis into a 2 ml. syringe and ejected from the syringe, 1 ml. into each of two vials. They were re-stoppered immediately and placed horizontally on a rotating wheel which revolved at a frequency of 40 R.P.M. At the end of thirty minutes' rotation, the vials were removed from the wheel and the glass rods plunged into 1.5 ml. of Nessler's solution (6) diluted 1:10 with ammonia free (Permutit treated) distilled water contained in 1 cm. cuvettes for the Coleman Junior Spectrophotometer. The stopper was pressed tightly against the mouth of the cuvette and the cuvette inverted to assure mixing. The optical density was read at the end of ten minutes at a wave length of 500 millimicrons. Duplicate standards were run simultaneously, and all values calculated on the basis of these standards. Duplicates of the blood samples were also run in all cases. Half milliliter aliquots of blood were used when the ammonia level was expected to be high (1 ml. syringe). No correction for diffusion difference between blood and standard was applied.

Patients were volunteers or comatose patients.

RESULTS

Table I-A gives the arterial and cerebral venous ammonia levels in 13 patients (15 observations) with a variety of conditions, not including any known hepatic disorder. Table I-B shows the arterial and peripheral venous ammonia levels in six patients without liver disease. It is clear that the blood levels of ammonia by this method cover only a narrow range and that normal individuals, or those not suffering from liver disease, have ammonia nitrogen levels of 1 microgram per ml. or less by this method.

Table II-A gives the arterial and cerebral venous ammonia levels in 13 patients (17 observa-

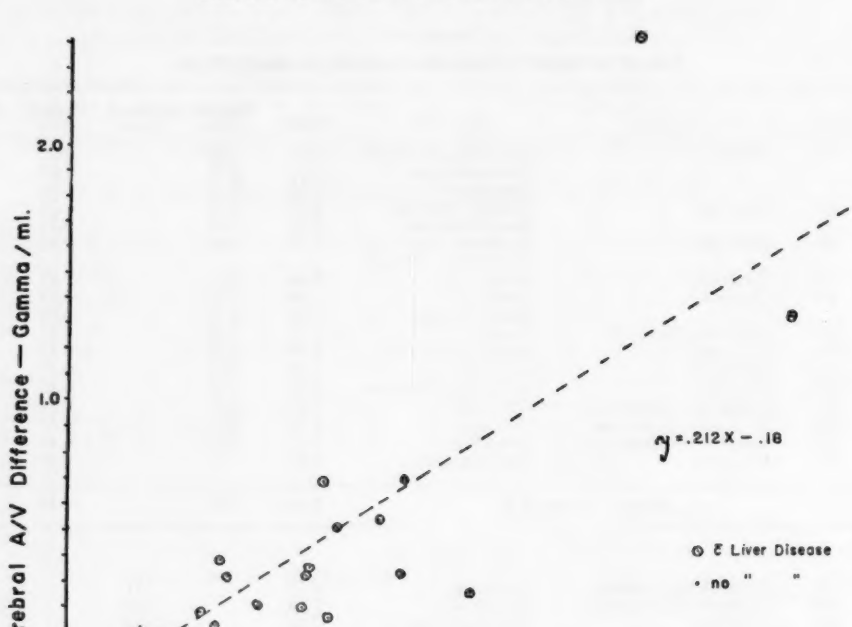

FIG. 1. THE RELATION OF THE CEREBRAL ARTERIOVENOUS DIFFERENCE OF AMMONIA TO THE ARTERIAL LEVEL

Correlation coefficient for these data is 0.82.

TABLE III

Liver function studies of patients in Table II

Pt.	Ceph. floc. *plus* (+)	Thymol turbidity (*units*)	Alk. phosph. (*Bodansky units*)	BSP* %	Van den Bergh Dir. (*mgm.* %)	Van den Bergh Indir. (*mgm.* %)	Tot. prot.	Alb. (*gm.* %)	Glob. (*gm.* %)	Ascites
1. T. B.	4	1		28	0.4	0.2				none
2. A. J.	4	3		31	0.8	1.2				1
3. H. A.	4	13		31						none
4. W. S.	4	5	1.6		10.6	6.4	8.5	3.1	5.4	3
5. L. P.	4	8			0.2	1.1	5.2	2.8	2.4	4
6. V. W.	4	16								2
7. L. G.	4	11			26	15	7.8	2.7	5.1	1
8. J. D.	4	9		26						3
9. W. M.	4	12		27						1
10. L. M.	4	10								none
11. L. H.	4	15		35	1.2	1.3				2
12. E. B.	4	9	10	29						3
13. R. L.	4	7.5			1.5	1.9	7.3	3.5	3.8	2
14. C. F.	4	8	9		6.8	13.8	6.8	3.6	3.2	2
15. J. S.	3	7	24	25			8.1	3.1	5.0	3
16. W. C.	3	9								none
17. R. W.	4	7	9		2.2	3.0	8.0	4.7	3.3	none

* BSP = Bromsulfalein retention at 45 minutes after 5 mgm. per kg. dose administered intravenously.

tions) with liver disease, both comatose and noncomatose. The last three cases of Table II-A are included as the first three cases of Table II-B. In these three cases, arterial, peripheral and cerebral venous blood samples were drawn within five minutes of each other and all measured for ammonia content. In all cases, whether the patient was comatose or not, the blood values for ammonia were elevated above normal. In no case was there a negative cerebral arteriovenous difference: the final figures in Table II-B give the average levels of the three patients in whom the arterial, cerebral and peripheral ammonia levels were measured simultaneously, and the values for peripheral and cerebral arteriovenous differences are nearly the same. The degree of coma, assessed clinically, is roughly proportional to the level of ammonia, and for two patients (T. B. and A. J.) the levels of ammonia parallel the development of coma. Figure 1 is a plot of all the arteriovenous differences of ammonia nitrogen of Tables I and II against the arterial blood level of ammonia nitrogen. The uptake of ammonia by the brain shows a striking correlation to the level of ammonia nitrogen entering the brain.

Table III presents various liver function studies done on patients in Table II. All these subjects had moderate to severe impairment of hepatic function by these various tests. However, no one test paralleled the mental state of the patient as closely as the arterial blood ammonia level.

DISCUSSION

The results presented confirm the recently reported observations of McDermott and Adams (2) who reported that the appearance of abnormal neurological signs shown by a patient with cirrhosis of the liver and Eck fistula paralleled changes in the blood ammonia levels.

The data reported here are also consistent with the data of Phillips, Schwartz, Gabuzda, and Davidson (3), if the differences in method are taken into consideration. The values reported by that group as normal (1.0 to 3.0 gamma per ml.) are far higher than the values of Conway (7), which might, from the standpoint of technique, be considered to be more accurate indices of the true blood ammonia. Our normal values fall close to the Conway range and never enter the range reported by the above (3) investigators. This suggests that the extraneous nucleotide ammonia, increasing amounts of which are determined as free ammonia as the diffusion time is prolonged, may mask changes in free blood ammonia which have profound significance in the development of coma. The five patients (A. D., J. M., C. J., E. B., and J. B.) on whom the above investigators (3) made determinations during "impending coma" and coma, show a mean increase from the first to the second state of 1.4 gamma per ml. Only one of them (C. J.) showed a fall in blood ammonia, and this was only 0.4 gamma per ml. which could lie within the experimental error of their method.

The positive A-V difference of free ammonia shown by the data presented in this report lead to the conclusion that free blood ammonia is converted by brain and muscle to a bound form which is no longer detectable by the method employed. This "utilization" of free ammonia by brain in hepatic coma suggests an hypothesis concerning the pathologic chemistry of this syndrome. The fact that ammonia is taken up by brain and that it is not in physical equilibrium with brain tissue, which would be the case if the arteriovenous difference were zero (as it is in patients without liver disease), indicates that ammonia is being utilized in some chemical reaction. Of the many reactions

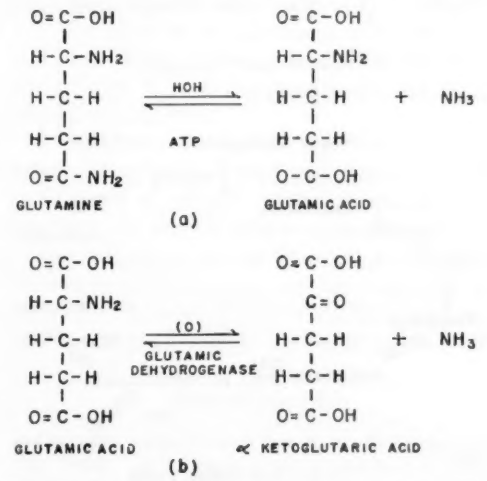

FIG. 2. THE MAJOR REACTIONS FORMING, OR UTILIZING, AMMONIA IN BRAIN

utilizing ammonia in the body, two are of significance in the brain—glutamine synthesis, and the reversal of glutamate oxidation, namely reductive amination of alpha ketoglutarate, Figure 2.

Glutamine synthesis could account for the high uptake of ammonia, and there is no doubt that at approximately 0.01 molar (8) there is sufficient glutamate in brain to supply a fair amount for the synthesis initially to take place. The requirement of ATP for the synthesis of glutamine might at first glance suggest that this could be the cause of the coma, by taking ATP from supplies necessary for other cerebral activities. This explanation is not, however, consistent with the mass of experimental data which shows that the removal of ATP from an oxidizing system does not inhibit the system, but rather stimulates it to further oxidative activity (9–11). Thus, if we are dealing merely with glutamine synthesis, we should find the oxygen uptake of the brain in hepatic coma increased. In addition, in spite of the preformed glutamic acid present in brain, it would not be long, even at a rate of utilization of ammonia of only 1 microgram per ml. to exhaust the total brain glutamate. (At a rate of cerebral blood flow of 55 ml. per 100 g. per min., such a difference would imply an uptake of almost four micromoles of ammonia per minute per 100 gm. or a loss of glutamate at the same rate.)

On the other hand, the synthesis of glutamate, Figure 2 (b), from alpha ketoglutarate could produce the phenomena seen in hepatic coma. To elucidate this more clearly we must refer to the Krebs cycle (Figure 3) which is the most likely final oxidative pathway in brain. The features of this cycle pertinent to this hypothesis are that the cycle is regenerative, requiring only minimal concentrations of any member at any time, and that alpha ketoglutarate is an essential member. It is apparent that any reaction which would tend to remove alpha ketoglutarate would also diminish the rate of formation of the succeeding members of the cycle. Since a major portion of the oxidative phosphorylation of brain is derived from the Krebs cycle, oxygen utilization and the formation of metabolic energy (high energy phosphate) will be diminished in direct proportion to the reduction in ketoglutarate available for the cycle. Both the decreased oxygen uptake and the utilization of ammonia by brain are part of the picture of hepatic coma. Wechsler, Crum, and Roth (12) report a greater depression of cerebral metabolism than of cerebral blood flow in hepatic coma and postulate that the "results implicate an intra cellular metabolic abnormality in the brain as a cause for the reduction in cerebral metabolism and coma in severe liver disease." Fazekas and Bessman (13) have found similar depression of cerebral oxygen consumption in patients who are semicomatose or comatose due to liver disease.

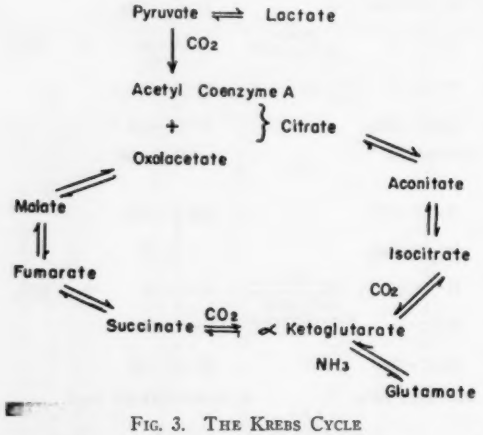

FIG. 3. THE KREBS CYCLE

A number of intermediate steps not pertinent to the discussion have been omitted.

The finding of some increase in the glutamate leaving the brain when ammonia is entering (1) is corroborative of the hypothesis that the lesion in ammonia intoxication is a reversal of the glutamic dehydrogenase reaction with resultant *formation* of glutamate by brain. Von Euler, Adler, Günther, and Das (14) have shown that the equilibrium for reaction II(b) is far to the left and that a very low concentration of ammonia can push the reaction over to net synthesis of glutamate.

This mechanism of action of ammonia has been studied by Potter and Recknagel (15) who showed that the *in vitro* inhibition of tissue respiration by ammonium chloride was accompanied by accumulation of glutamic and aspartic acids. The further possible effects of a glutamic acid excess so produced are suggested by experiments reported from this laboratory (16, 17).

The uptake of ammonia by muscle is to be expected in view of the fact that the Krebs cycle is operative here as well as in brain, and that there

is an active glutamic dehydrogenase system in muscle.

Although the evolution of ammonia from muscle on exertion has been traced to deamination of adenine nucleotides (18), no comparable study has been made of the uptake of ammonia by muscle when the blood ammonia is elevated. It is of clinical significance in two ways. It explains the high level of glutamate found in the blood of patients with hepatic coma (19). In addition, it suggests that in the presence of an elevated arterial ammonia, the measurement of venous ammonia gives a false value for the ammonia level in the blood reaching the brain. This difference between arterial and peripheral venous ammonia would be of qualitative importance in the understanding of borderline cases since there would conceivably be sufficient uptake of ammonia by muscle to mask an elevated arterial level, and of quantitative significance in comparative studies of blood ammonia as related to clinical phenomena of hepatic coma.

A word might be said relative to a possibly broader significance of blood ammonia in the coma and cerebral symptoms of other diseases. For example, Nelson's and Seligson's extensive study of blood ammonia in experimental shock (20) clearly demonstrates an uptake of ammonia by brain. The high levels of glutamic acid found in the blood of patients with many types of wasting disease, particularly malignancy (21), suggest a possibly fruitful investigation of the relation of ammonia to cerebral symptoms in these diseases. Preliminary studies in our laboratories reveal elevated blood ammonia levels in patients with cardiac failure, and may implicate ammonia in the cerebral symptomatology of cardiac (22) disease.

From the standpoint of therapy there are several factors which must be considered. It has been reported that the blood level of alpha ketoglutaric acid is increased in hepatic coma (23). At first glance this would seem incompatible with any theory which postulated diminished levels of this intermediate in the brain. Alpha ketoglutarate, however, being a dibasic acid, is not taken up by the brain, and lack of this compound could well exist behind the "blood brain barrier." This keto acid could be a therapeutic agent and probably does act in muscle to ameliorate the high levels of blood ammonia by the same reaction which is excessive in brain, namely, the reductive amination to glutamate. The high levels of blood ketoglutarate probably result from the inability of the damaged liver to oxidize the intermediate products of fat, protein, and carbohydrate completely.

The use of glutamate in therapy of hepatic coma, as suggested by Walshe (24), could be rationalized on the basis of glutamine synthesis, Figure 2, which goes on in kidney and liver, as well as brain. This might indeed be a good basis for its use, but clinical experience has not been uniformly favorable (25).

The use of substances designed to reduce the *production* of ammonia might offer some promise. This was accomplished by McDermott and Adams (2) by the administration of non-absorbable sulfonamides, which probably had the effect of altering the intestinal flora, with loss of ammonia forming organisms.

SUMMARY AND CONCLUSIONS

1. Arterial and cerebral venous blood ammonia determinations are reported in normal patients, patients with hepatic disease, and in a variety of diseases in the presence and absence of coma.

2. In hepatic disease, the blood level of ammonia is uniformly elevated, and is roughly proportional to the depth of coma.

3. The brain takes up ammonia when the blood level rises above 1 gamma per ml. The uptake of ammonia by brain is roughly proportional to the arterial concentration.

4. Simultaneous determinations of peripheral venous blood with arterial and cerebral venous blood show a parallel uptake of ammonia by muscle as the arterial level of ammonia rises.

5. A hypothesis for the chemical pathology of hepatic coma is presented, and the suggestion offered that the phenomenon of ammonia intoxication is a part of a number of other syndromes.

ACKNOWLEDGMENT

The authors wish to thank Drs. W. V. McDermott and Ray Adams for the opportunity to discuss their observations prior to publication (2). The work reported and the hypothesis presented are the outgrowth of these stimulating conversations.

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THE POTENTIAL LIFE SPAN AND ULTIMATE SURVIVAL OF FRESH RED BLOOD CELLS IN NORMAL HEALTHY RECIPIENTS AS STUDIED BY SIMULTANEOUS Cr^{51} TAGGING AND DIFFERENTIAL HEMOLYSIS [1]

By G. S. EADIE and IVAN W. BROWN, Jr. with the technical assistance of W. GRANDY CURTIS

(From the Departments of Physiology and Pharmacology and of Surgery, Duke University School of Medicine, Durham, N. C.)

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Death of the red blood cell is thought to occur either by senescence when it reaches the end of its life span or by a process of random destruction unrelated to the age of the cell (1, 2). In a normally distributed mixed population of red cells senescence would result in the daily loss of approximately the same number of erythrocytes while random destruction would remove each day a constant proportion of the remaining cells. These two processes are therefore clearly distinguishable by survival experiments. Although this theory implies that the life span, as distinct from the actual survival time, is a characteristic of the cell itself and independent of the environment so that it is unaffected even by transfusion, no direct experimental evidence exists as to whether or not this is, in fact, the case. To obtain such evidence it is necessary to use cells that can be identified either by the blood group to which they belong as in the Ashby method, or by the incorporation of an isotope such as N^{15}, Fe^{55}, Fe^{59}, or Cr^{51}. Identification with radiochromium is perhaps the simplest because of the ease of incorporation and of measurement, its greatest defect being that its union with the red cell is not quite irreversible, approximately 1 per cent of the remaining isotope being lost, presumably by elution, daily (3). This makes direct calculation of the life span and rate of random loss impossible by the usual regression methods, and although a method exists by which an approximation can sometimes be arrived at, it is not certain whether this would give satisfactory answers in this case. Moreover very little is known about the accuracy and reproducibility of determinations of life span made by any method, and it was hoped that information might be gained on this point also.

METHODS

Two experiments were performed. In each a large volume of blood, a liter or more, was drawn from a healthy male donor belonging to group O, divided into three parts, and transfused into three healthy, compatible, male recipients belonging to group A, sterile precautions being used throughout. The fate of the transfused blood was followed by two different methods: by tagging the cells with radiochromium, and by a modification of the Ashby method described below using differential hemolysis instead of agglutination.

Donor blood was drawn into plastic bags containing 75 ml. of Anticoagulant Acid Citrate Dextrose Solution (Solution A), U. S. P. After thorough mixing about 50 ml. were removed and incubated at room temperature for 45 mins. with a sterile solution of sodium chromate containing 50 to 100 microcuries of Cr^{51}. The specific activity was 862 millicuries per gm. of chromium in the first experiment and 1,358 millicuries per gm. in the second. The cells were then centrifuged, washed three times with ice-cold saline, and finally suspended in saline. The hematocrit values of this mixture were determined by a procedure previously used in this laboratory which consists of centrifuging the blood at 3,000 rpm. for 45 mins. and correcting the resulting figure for the 3 per cent of the saline that had been shown to be trapped among the corpuscles under our conditions (4). The radioactivity of the red cell suspension was measured in a well type scintillation counter [2] after diluting 1:100 with distilled water. Meanwhile, immediately before transfusion, about 300 ml. of blood was removed from each recipient by venipuncture so that the subsequent blood infusion would not produce any great change in the total red cell mass. Accurately measured samples of approximately 10 ml. of the well mixed blood treated with Cr^{51} were then injected by calibrated syringes into each recipient and samples taken 10 and 20 mins. after injection. From the radioactivity and hematocrit value of these samples, the total circulating red cell mass was

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[2] Obtained from N. Wood Counter Laboratories, 5491 Blackstone Avenue, Chicago, Illinois.

TABLE I

Donor	Recipient	Vol. of injected chromated red cells ml.	RBC volume of recipient liters	Vol. of injected non-chromated red cells ml.
I	1	3.71	1.949 av. 1.918 1.887	145.2
I	2	3.68	1.865 av. 1.984 2.104	98.6
I	3	3.59	1.916 av. 1.954	123.5
II	4	3.98	1.837 av. 1.862 1.887	164.7
II	5	3.93	1.324 av. 1.324 1.323	153.9
II	6	4.00	1.814 av. 1.811 1.808	165.0

calculated (Table I). Following this each recipient received 250 ml. to 350 ml. of chromium-free blood of which the exact volume had been determined by weighing and measuring the specific gravity. In addition, the hematocrit value, and, in the case of the second donor, the red cell count of the transfused blood, were also determined. It should be noted that the volume of chromate-free blood was very much greater than that of the chromium-tagged blood; most of the cells followed by differential hemolysis had not been exposed to chromium, and any possible effect of radiochromium on survival would be largely avoided.

Some thirty samples for analysis were obtained from each recipient over a period of about 100 days spaced as follows. Two samples were taken the first day, one 30 mins. and the other two hours after transfusion. Following this samples were taken daily for a week, and thereafter twice a week. In each case blood was collected in tubes containing 0.2 mg. of dried heparin. Radioactivity was determined on each sample, diluted if necessary with distilled water, and was corrected for the decay of radioactivity of the chromium itself.[3] The activity in counts per minute was then divided by the hematocrit reading and expressed as cpm per ml. of red cells. Multiplication by the previously determined red cell mass gave the total circulating radioactivity assuming no change in red cell mass. In this form it was possible to correct for loss of chromium in the samples by using a formula previously given (4).

[3] In order to have a check on the scintillation counter readings the activity of a sample of the radiochromium was measured at the same time as the blood samples. From these readings the half-life was calculated to be 27.4 and 27.3 days. This is to be compared with the value of 27.75 ± 0.3 days given by Lyon (6). Measurements were therefore reasonably accurate.

The number of circulating donor cells was counted by a method of differential hemolysis as follows.[4] Human anti-A hemolytic serum was obtained from a group B volunteer who had been injected with 5 ml. of Blood Group Specific Substances A and B Solution (Sharp and Dohme) once weekly for three weeks. To this, fresh complement was added in the form of guinea-pig serum. For this purpose guinea-pigs were chosen which had a natural anti-A hemolysin in their sera (5); other animals were found to be entirely unsatisfactory. The guinea-pig anti-A serum titer was enhanced by intraperitoneal injections of a mixture of 10 parts of sterile meconium from a newborn group A secretor infant, one part of Arlecel A[5] and nine parts of Bayol F[6] (8). The human and guinea-pig sera were mixed in equal volumes, other proportions being found to be less effective. They were, of course, tested to ensure absence of species specific hemolysins and hemolysins for group O cells.

Blood to be counted was diluted with saline in the ratios 1:25, 1:50 or 1:100 (or 1:26, 1:51 or 1:101) depending on the number of donor cells anticipated; this was done in order that approximately the same number of cells might be counted throughout the experiment without unduly increasing the number of spreads. This

[4] Since the completion of this work a method of differential hemolysis has been published by Hurley and Weisman (7). Their method differs from ours chiefly in the use of the recipient's serum rather than guinea-pig serum as a source of complement. By using the guinea-pig serum it was possible to make counts at lower blood dilutions with greater reproducibility.

[5] Obtained from Atlas Powder Company, Wilmington, Delaware.

[6] Obstained from Esso Standard Oil Company, Bayonne, New Jersey.

helps to minimize the decrease in accuracy of counting which would otherwise occur towards the end of the experiment with the decrease in the numbers of donor cells. The diluted cell suspension was mixed with an equal volume of the hemolytic antiserum mixture, a proportion found experimentally to be the most satisfactory, and incubated in a water bath at 37° C. for 30 mins. with occasional shaking. Counts were made by a photographic method (9). While this hemolytic method is not completely free from various errors, nevertheless it is our experience that results obtained with it are much more accurate than with the usual differential agglutination procedures (10). Counts expressed as millions of cells per cu. mm. were divided by the hematocrit reading expressed as a decimal rather than a per cent and multiplied by 100 to avoid small numbers. For example a count of 0.450 million cells per cu. mm. and a hematocrit reading of 0.45 (45 per cent) would give a nonhemolyzed cell index of 100. This gives the count in terms of the red cell mass rather than the blood volume and thus avoids errors due to fluctuations in the latter.

RESULTS AND DISCUSSION

Difficulties in interpretation of curves obtained with radiochromium stem primarily from the fact that some chromium leaves the red cell before its destruction probably by elution (3); this is thought to occur at a rate proportional to the average concentration of chromium remaining in the erythrocytes at that moment, and independent of the age of the cell, *i.e.*, at a rate equal to k_1p where p is the corrected radioactivity on a given day. In addition to this, radiochromium is lost when cells disappear for any reason, whether by senescence or random destruction. Since the latter occurs at a rate equal to k_2p, its rate may be added to that of elution to give $(k_1 + k_2)p$, and we may substitute k for $k_1 + k_2$. The constant k_2 of course in man is frequently zero, *i.e.*, there is no random destruction, and in this case k will equal k_1.[7] Because the rates of random loss may be added in this way it is possible to describe the disappearance of radiochromium from circulating blood by the equation (2, 11)

$$N = N_o(1 - t/T)e^{-kt},\ t \leqq T \qquad (1)$$

where N is the radioactivity count, corrected as described under Methods, on day t, N_o the corresponding count on day zero, and T the potential life span. In dealing with radioactive decay it is customary to give the half-life rather than the coefficient of decay, λ. These are, however, readily convertible since $0.5 = e^{-\lambda t}$ leads to $\lambda = -0.693/t$, t in this instance being the half-life. The situation is different with red cell disappearance where there are two parameters, T and k, instead of one. There is consequently an infinite number of pairs of values of T and k for each value of the half-life which is therefore ambiguous. Finally, it should be noted that this equation assumes that chromium set free by elution or by destruction of the red cell with which it is combined, is not used for labeling other red cells. This is in agreement with the findings of Ebaugh, Emerson, and Ross (12).

Fitting data to equation (*1*) is difficult because it is impossible to reduce it to a straight line by using logarithms or other devices; and although data for the first two or three weeks plotted in this way do not depart greatly from linearity, the error is sufficient to make estimates of T and k unreliable and that of N_o somewhat too high. Assumption of a value for T permits evaluation of k (13), but this is biased by the assumption with regard to T. An approximation to a least squares solution may, however, be made by a method given by Deming (14). Values are assumed for the three parameters of equation (*1*) N_o, T and k; these are inserted into the expression

$$N - N_o(1 - t/T)e^{-kt} \qquad (2)$$

and into the expressions obtained by its partial differentiation with respect to each of the three constants; this is done for each value of t for which experimental data exist. Four columns of figures are thus obtained; from the sums of their squares and cross-products three normal equations are formed whose solution (preferably by the methods of matrix algebra) furnishes three numbers which, subtracted from the assumed values of the constants, give the first approximations to an unbiased estimate. Repetition of this procedure would yield a closer approximation, but this did not seem necessary in the case of any of the experiments reported here. Standard errors are calculated by determining the inverse matrix. The weighting procedure suggested by Deming seemed to be unnecessary since counts were made in such a way

[7] Decay of radioactivity follows the same law as random loss, and the radioactivity decay constant, λ, may also be added and $k' = (k_1 + k_2 + \lambda)$ may be substituted for *k* in the equation above instead of correcting the counts for decay as described above.

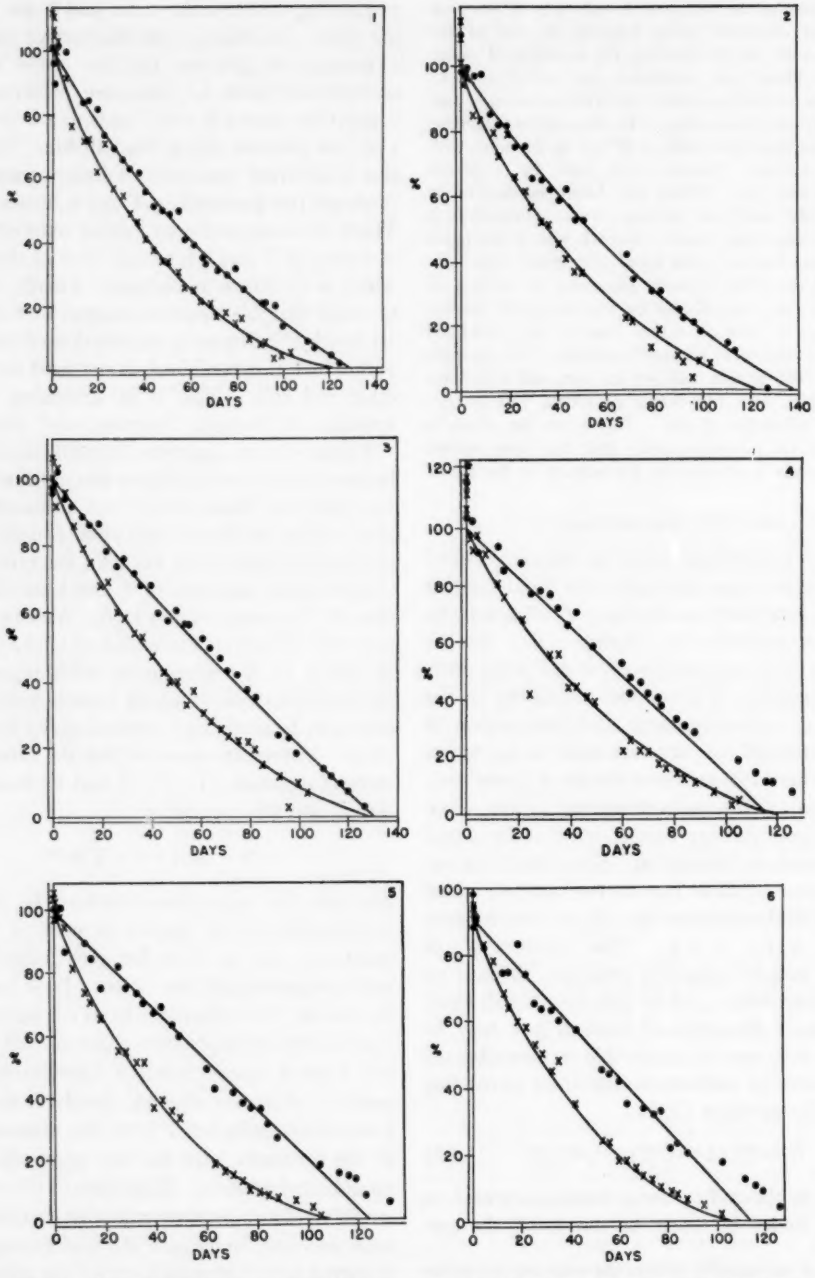

FIG. 1. SURVIVAL OF ERYTHROCYTES TRANSFUSED FROM HEALTHY DONORS INTO HEALTHY RECIPIENTS FOLLOWED SIMULTANEOUSLY BY DIFFERENTIAL HEMOLYSIS AND TAGGING WITH RADIOCHROMIUM

The index of non-hemolyzed cells (●) and corrected radioactivity (×) are plotted on a scale where 100 per cent represents the value of N_o given in Table II, *i.e.*, 100 per cent of the

that the total number of radioactive counts or of red cells enumerated were approximately the same throughout the experiment, and accordingly it was not used. Counts made on the day of transfusion were not used in order to avoid effects from the rapid elimination of any damaged cells (2).

From the values of N_0, T and k obtained by this analysis a disappearance curve was constructed for each recipient. These together with the data from which they were derived, are plotted in Figure 1. It is obvious that except for the initial and final periods the fit is very good and the experimental points lie randomly on either side of the line. There is thus no reason to think that the equation fails to represent adequately the rate of cell disappearance over the major part of its course, nor is there any evidence of relabeling by released chromium for the metal does not persist in the blood after transfused cells have been shown to disappear by the method of differential hemolysis. Departures are visible towards the end of the curves in the second experiment (Figure 1, curves 4, 5, and 6). This is particularly noticeable in the hemolysis data (solid circles). This is usually ascribed to the fact that life spans of individual cells are not all precisely equal but distributed around a mean (1, 2). Why this should not also be apparent in the first experiment (Figure 1, curves 1, 2, and 3) is not clear, but there is no doubt of the absence of tailing throughout this group of curves. Because large experimental errors are commonly found in this part of the curve, it has not always been possible in the past to be certain of the presence or absence of such tailing, but with the increasing accuracy of methods now available it can be studied more carefully. If it should be found that a tail is only occasionally present, some revision of the present theory will probably be necessary.

Values for N_0, T and k obtained by the analysis are summarized in Table II. It will be seen that there is a considerable degree of variation in estimated life spans even for samples of blood from the same donor, and that differences also occur between estimations made by different methods from the same recipient. Whether these differences are significant cannot be ascertained by the usual tests because of the wide range of variation in the standard errors. Bartlett's test (15) shows that the variance is not homogeneous in either experiment when cells were tagged with chromium, and was very questionably so with the hemolytic method in the first experiment. Comparisons were therefore made by a method based on Fisher's theory of fiducial probability (16), and also by the approximation suggested by Cochran and Cox (17). Neither of these tests showed a significant difference in values for potential life span attributable to the method or to the recipient. It may be noted, however, that values obtained by the radioisotope method tend to be lower than those obtained by hemolysis; the difference, while it does not reach the level of significance with the few data available, nevertheless tends to approach it, and might very well prove to be significant in a longer series. But, even if this were the case, the reason for it might quite as likely be a systematic experimental error in one of the methods as a change in life span. However this may be, it is quite clear that chromium has no significant deleterious effect at least on the life span.

Because of this absence of significant differences among various determinations on blood from the same donor, it was possible to pool data. For the first experiment the average life spans proved to be 128.7 days with a standard deviation of 4.6; for the second experiment the figures were 114.0 $\pm$ 7.7. The two variances are homogeneous, and the two values for the life span differed to a highly significant degree. The potential life span therefore differs depending on the donor and is independent of the environment.

The lack of uniformity in variance, already mentioned, appears, in the first experiment, to be related to the coefficient of random loss, but this is not the case in the second. It would seem reasonable to think that determinations made from data collected over a shorter period would show a

surviving cells rather than the total number of cells injected. Abscissas give the time in days. The smooth curves were drawn from the equation in the text using the values for the constants in Table II. Each pair of curves was obtained from the same recipient (the number in the right upper corner is the recipient's number). Blood from the same donor was used in curves 1, 2, and 3 and another in curves 4, 5, and 6.

TABLE II*

Expt.	Recipient	Method	df	T	k	N_o
1	1	a NHC	29	129.29 ± 3.61	.00461 ± .00073	100.30 ± 1.56
		b Cr	28	125.08 ± 11.85	.0125 ± .0041	3.35 ± .06
	2	a NHC	23	137.01 ± 3.57	.00404 ± .00071	81.65 ± 1.09
		b Cr	22	124.43 ± 10.92	.00978 ± .00161	3.39 ± .073
	3	a NHC	29	129.25 ± 2.06	.00069 ± .00046	86.06 ± .91
		b Cr	27	127.07 ± 5.15	.00915 ± .00281	3.46 ± 0.27
2	4	a NHC	19	116.79 ± 7.01	.00100 ± .00128	81.77 ± 1.61
		b Cr	24	118.33 ± 13.40	.00994 ± .00204	5.343 ± .014
	5	a NHC	19	123.86 ± 7.77	.00056 ± .00133	120.72 ± 2.14
		b Cr	23	108.70 ± 8.23	.01199 ± .00146	5.908 ± .088
	6	a NHC	18	114.22 ± 6.74	.00044 ± .01724	99.86 ± 2.63
		b Cr	21	102.10 ± 5.84	.00992 ± .00105	5.954 ± .078

*df: degrees of freedom, in this case equal to the total number of observations less 3.

T: The average potential life span with its standard error. The latter indicates the precision of the determination, not the spread of life spans around the mean.

k: the fraction of cells disappearing daily by random destruction and the standard error of its determination.

N_o: value for day zero by extrapolation, with the standard error of its determination. The difference between this and the expected value would indicate the number of irreversibly damaged cells in the transfusate.

greater variance than those collected over a longer one, but the data do not confirm this, again particularly in the second experiment.

Random loss, in the case of chromium experiments, includes both elution and random destruction, and k is therefore greater than in the hemolytic experiments where elution is absent. It was somewhat surprising to find that k was not always zero in the latter experiments, indicating the presence of random destruction. The cause of this was not established. Since some of the cells counted by the hemolytic technique had been treated with Cr^{51}, the possibility of a toxic effect of chromium must be considered. The proportion of treated cells injected, however, never exceeded 4 per cent of the total; and any effect from this source would therefore be insignificant. Moreover, assuming that the random loss in Expt. 1.1.a (Table II) is due to random destruction as a result of the action of Cr^{51} on some of the cells, we may calculate that if all the cells had been so treated the rate of destruction would be greater by a factor equal to the increase in the proportion of cells treated. That is since the value of k in the hemolytic method was 0.0046 with 2.6 per cent of the cells treated with Cr^{51} the expected value of k with 100 per cent of cells treated would be $0.0046/.026 = 0.177$. This is much more than ten times the value actually found, *viz.*, 0.0125. Damage from chromium is therefore highly improbable. In the greater number of experiments random destruction as measured by the hemolytic method is not significantly greater than zero in spite of the fact that some of the cells had been exposed to chromium, which therefore cannot in these cases have been the cause of any random destruction. The recipients remained in excellent health throughout the experimental period, and no detectable incompatibility between donor and recipient blood was found either before or after the experiment by multiple compatibility tests including the indirect antiglobulin test (Coombs). De-

velopment of an antibody during the course of the experiment is also very unlikely since the rate of random destruction is, as closely as one can judge, constant throughout. One must conclude that even in the blood of so-called normals there may be present a mechanism of unknown nature causing random loss of red cells. That this has not previously been noted in man is probably to be explained by its being obscured by the experimental errors involved in the Ashby method. It has, however, uniformly been seen in apparently healthy cats and rabbits, and also, although only occasionally, in dogs when blood was tagged with Fe^{59} (4). In the remainder of the counts made by the hemolytic method k was not significantly greater (or, in one instance, less) than zero, that is to say, there was no random destruction, but only disappearance as a result of senescence.

The increased rate of calculated random loss in the chromium experiments is probably to be attributed to elution. It is measured by the differences in constants of random loss obtained by the chromium and hemolytic methods; values are given in Table II. Standard errors are computed by taking the square root of the sum of the squares of the standard errors of the two k's. Because of the large standard error it was impossible to show significant differences between different recipients. The average daily rate of loss was .0090. This might result either from a toxic effect of the chromium or from elution, or even as Ebaugh, Emerson, and Ross (12) suggest, unequal effects of chromium on cells of different ages. This last would imply that on any day a red cell would take up 0.9 per cent less chromium than it would have done the day before, an assumption for which there is no other evidence. Nor is there any independent reason for attributing it to a toxic effect of the isotope. It is clearly unrelated to the specific activity of the chromium used, and Ebaugh, Emerson, and Ross were unable to relate it to chromium concentrations. On the other hand the figure 0.9 per cent is very close to the value of 1 per cent which Necheles, Weinstein, and LeRoy (3) found for elution, to which it may therefore be most probably ascribed. This would represent a biological half-life of 77 days which is exactly the same as that computed by Ebaugh, Emerson, and Ross (12).

Finally, analysis of the curve gives a value for the initial reading, N_o. It is interesting to compare this with values actually obtained within the first 24 hours, values which do not enter into the determination of this constant. With one exception (2.4.a Table II) values determined by hemolysis are scattered around the line; in all the radiochromium curves and one of the hemolytic curves the initial values lie above the line, often dropping sharply. Ebaugh, Emerson, and Ross (12) found that the chromium curve was 6.7 per cent, on the average, above the curve determined by agglutination during the first 48 hours. Similar observations have been made by Mollison and Veall (18), who found a loss of chromium of about 6 per cent in the first 24 hours. Although this may be due to more rapid elution, these facts might also be explained by assuming that the chromium treatment produces irreversible damage in a few of the cells treated; these disappear for the most part within twenty-four hours after transfusion. In this respect the damage resembles that produced by storage procedures, and in all these cases it may be the result of the manipulations such as centrifuging, transfusing, *etc.*, and not due to the chromium itself. It would not therefore be surprising to find it also in one of the curves obtained by differential hemolysis (2.4.a Table II). That it occurs so frequently means that considerable caution must be used in interpreting this portion of the curve. Apart from this there is no real evidence that chromium has any deleterious effect on the survival of the cells in which it has been incorporated.

SUMMARY

In order to distinguish between the innate life span of erythrocytes and environmental factors affecting their survival, red cells from the same donor were followed after transfusion into three different healthy recipients in each of two experiments. In every case the number of surviving cells was estimated by two methods: tagging with radiochromium and a new modification of the Ashby method using hemolysis instead of agglutination. Details of this method are given. Curves obtained by both methods were analyzed by a mathematical method not previously used for this work, and it was possible to obtain relatively precise estimates

of life span and coefficient of random destruction, together with their standard deviations. The life span, 129 ± 5 days in one case and 114 ± 8 in the other, was found to depend solely on the donor and to be independent of environment and the method. Random loss of radiochromium, presumably from elution, averaged 0.9 per cent daily; differences due to environment were not detectable. Random destruction of significant magnitude occurred in two of the six healthy recipients; it was not a toxic effect of the chromium, nor could antibodies capable of causing it be demonstrated. By projecting the curve obtained by this mathematical analysis backwards to zero time it was found that in one of the hemolytic curves and all of the radiochromium ones there was a definite drop in the count over the first twenty-four hours unaccounted for by elution (unless at a much greater rate during this period), random destruction or senescence. It was suggested that it was a result of damage to the blood preparatory to and during transfusion. No toxic effects of radiochromium were found.

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BLOOD FLOW THROUGH EACH LUNG IN MAN DURING UNILATERAL HYPOXIA[1]

By A. P. FISHMAN,[2] A. HIMMELSTEIN, H. W. FRITTS, JR.,[3] AND A. COURNAND

(From the Department of Medicine, Columbia University, College of Physicians and Surgeons, and the Cardio-Pulmonary Laboratory of the First Medical and Chest Services (Columbia University Division), Bellevue Hospital, New York, N. Y.)

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It is well established that pulmonary hypertension may be elicited in man by reducing the oxygen content of the inspired air. The mechanisms involved in this response to acute hypoxia, and the role of the individual segments of the pulmonary vascular tree in effecting this rise in pulmonary arterial pressure, are currently under investigation. In order to further analyze this phenomenon, a method has been developed for the separate measurement of blood flow through each lung in man. This method consists of a combination of (a) bronchospirometry, with each lung breathing a specifically selected oxygen mixture, (b) cardiac catheterization, and (c) arterial cannulation; this makes possible the application of the Fick principle, not only for the measurement of total blood flow, but also of blood flow through each lung.

Previous attempts to partition pulmonary blood flow in man have been indirect, and have involved many assumptions (1). It is the purpose of this paper to 1) describe a method based on more direct data, and 2) present the results of studies concerning the effects of unilateral hypoxia upon the pulmonary circulation in man.

METHODS

Principle of the method

The principle of the method may be outlined as follows: a) Oxygen uptake by each lung is determined by bronchospirometry; b) the oxygen content of the mixed venous blood going to both lungs is determined by sampling through a catheter placed in the pulmonary artery; c) the oxygen content of peripheral arterial blood, representing a mixture of blood leaving both lungs, is determined by analysis of a sample obtained through the indwelling arterial needle; d) the oxygen content of pulmonary venous blood leaving one of the two lungs, hereafter designated as lung H, is fixed at 100 per cent of capacity by the breathing of a gas mixture of oxygen in nitrogen somewhat higher in oxygen content than ambient air. This inspired gas mixture is capable of fully saturating with oxygen the hemoglobin in pulmonary venous blood, without unduly increasing the amount of oxygen carried in physical solution. The amount of oxygen in solution in the blood of a normal subject while breathing a mixture of 33 per cent oxygen in nitrogen will be approximately 0.5 ml. per 100 ml., and while breathing 25 per cent oxygen in nitrogen will be 0.3 ml. per 100 ml. These figures correspond within 0.2 ml. per 100 ml. to the amount of oxygen in physical solution when arterial blood is equilibrated *in vitro* with ambient air. Hence, the oxygen capacity of the arterial blood ($CAPa_{O_2}$) is substituted for the oxygen content of venous blood leaving lung H ($C_{PV_{O_2}} = CAPa_{O_2}$).

With the data thus obtained it is then possible to calculate 1) the minute volume of blood flowing through both lungs ($\dot{Q}_T$), and 2) the minute volume of blood flowing through lung H, ($\dot{Q}_H$), by employing two separate equations based on the Fick principle. The minute flow ($\dot{Q}_L$) through the other lung (lung L), which breathes either ambient air or a mixture of oxygen in nitrogen lower than 21 per cent, is then calculated as the difference between $\dot{Q}_T$ and $\dot{Q}_H$. *In contrast to indirect methods previously employed, no attempt is made to estimate the oxygen content of the pulmonary venous blood* ($C_{PV_{O_{2L}}}$) *of the hypoxic lung.*

The formulae used and their application to the problem of unilateral hypoxia, are illustrated in Figure 1.

Calculations and their justification

1. *Total pulmonary blood flow.* Total pulmonary blood flow per minute ($\dot{Q}_T$) is equal to the total oxygen uptake of both lungs per minute ($\dot{V}_{O_{2H}} + \dot{V}_{O_{2L}}$), divided by the corresponding arterial-mixed venous blood oxygen difference ($Ca_{O_2} - C\bar{v}_{O_2}$). This differs from the usual method of determining pulmonary blood flow only in that oxygen uptake for each lung is determined separately, and then summated.

2. *Blood flow through the lung breathing the higher oxygen mixture (lung H).* Since the oxygen uptake of the high-oxygen lung per minute ($\dot{V}_{O_{2H}}$), and the oxygen content of mixed venous blood ($C\bar{v}_{O_2}$) have been determined in the process of measuring *total* minute pulmonary blood flow

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[2] Established Investigator of the American Heart Association.

[3] Fellow, Life Insurance Medical Research Fund.

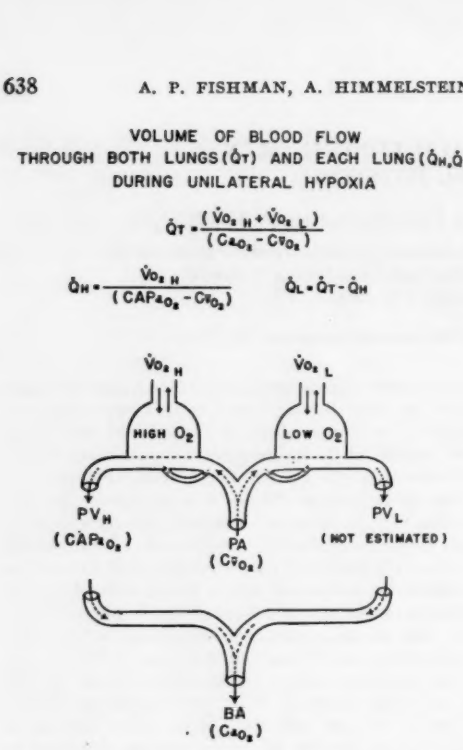

FIG. 1. SCHEMATIC REPRESENTATION OF THE METHODS AND FORMULAE FOR THE DETERMINATION OF TOTAL AND UNILATERAL PULMONARY BLOOD FLOWS

For description, see text.

($\dot{Q}_T$), the only remaining unknown for the calculation of blood flow through the high-oxygen lung per minute ($\dot{Q}_H$) by the Fick principle, is the oxygen content of pulmonary venous blood leaving this lung ($C\bar{P}v_{O_{2H}}$).

It was demonstrated on the day before the study, by brachial artery puncture, direct sampling, and chemical analysis for oxygen content and capacity of whole blood, that breathing a mixture of 25 per cent oxygen in nitrogen resulted, in all but one of our subjects, in an oxygen saturation $\left(\frac{O_2 \text{ content}}{O_2 \text{ capacity}} \times 100\right)$ of peripheral arterial blood, of not less than 99 per cent, and not greater than 100 per cent. In only one subject was it necessary to resort to inspired gas mixture containing 33 per cent oxygen in nitrogen to obtain such an oxygen saturation. After the preliminary determination of the composition of the inspired gas mixture necessary to accomplish this degree of arterial blood oxygen saturation, the mixture was subsequently administered *via* bronchospirometry tube, on the day of the experiment, to lung H as its inspired gas; the oxygen capacity of the arterial blood (CAP_{aO_2}) was hereafter substituted as equivalent to the oxygen content of pulmonary venous blood leaving lung H ($C\bar{P}v_{O_{2H}}$).

3. ***Blood flow through the lung breathing the lower oxygen mixture*** ***(lung L).*** As indicated above, blood flow through this lung per minute ($\dot{Q}_L$) is determined as the difference

TABLE I

Blood gas composition and gas exchange in the lungs during unilateral hypoxia

Subject	State	F_{IO_2} Lung H	F_{IO_2} Lung L	Blood gas composition Ca_{O_2} ml./100 ml.	Art. HB_{O_2} %	$C\bar{v}_{O_2}$ ml./100 ml.	Gas exchange in lungs $\dot{V}_{EH}$ L./min.	$\dot{V}_{EL}$ L./min.	$\dot{V}_{ET}$ L./min.	$\dot{V}_{O_{2H}}$ ml./min.	$\dot{V}_{O_{2L}}$ ml./min.	$\dot{V}_{O_{2T}}$ ml./min.	$\dot{V}_{CO_{2H}}$ ml./min.	$\dot{V}_{CO_{2L}}$ ml./min.	$\dot{V}_{CO_{2T}}$ ml./min.	R_{E_H}	R_{E_L}	R_{E_T}
H. J.	Control	.25	.21	16.2	97	12.6	7.95	4.92	12.87	137	84	221	133	75	208	.97	.90	.94
	Hypoxic	.25	.12	15.2	92	11.8	6.50	4.79	11.29	166	65	231	124	68	192	.75	1.04	.84
D. C.	Control	.25	.21	17.4	98	14.1	8.09	6.06	14.15	156	107	263	145	113	258	.93	1.00	.98
	Hypoxic	.25	.10	16.8	93	13.5	7.05	5.28	12.33	189	82	271	142	108	250	.75	1.32	.93
G. H.	Control	.25	.21	17.7	97	13.5	7.35	6.48	13.83	142	142	284	130	144	274	.92	1.01	.96
	Hypoxic	.25	.10	16.2	90	11.1	7.90	6.09	13.99	218	93	311	132	144	276	.61	1.55	.89
P. T.	Control	.25	.21	17.2	99	14.3	7.01	5.59	12.60	167	122	289	144	104	248	.86	.85	.86
	Hypoxic	.25	.10	15.2	87	12.0	6.61	5.63	12.24	234	73	307	149	111	260	.64	1.52	.85
J. S.	Control	.33	.21	18.1	99	14.3	4.87	4.43	9.30	192	111	303	124	99	223	.65	.89	.74
	Hypoxic	.33	.10	17.6	94	14.0	5.26	4.80	10.06	233	67	300	128	95	223	.55	1.42	.74
R. R.	Control	.25	.21	22.7	99	18.0	7.22	5.61	12.83	189	155	344	174	108	282	.92	.70	.82
	Hypoxic	.25	.10	21.1	91	16.7	6.26	4.35	10.61	246	86	332	154	107	261	.63	1.24	.79
Mean	Control				98		7.08	5.52	12.60	164	120	284	142	107	249	.88	.89	.88
	Hypoxic				91		6.60	5.16	11.76	214	78	292	138	106	244	.66	1.35	.84

between total pulmonary blood flow ($\dot{Q}_T$) and blood flow through the high-oxygen lung ($\dot{Q}_H$).

Experimental procedure and subjects

The experimental procedure was attempted nine times; six studies were successfully completed. Our observations were confined to male subjects with sharply circumscribed, unilateral pulmonary lesions, *i.e.*, either arrested minimal pulmonary tuberculosis prior to segmental resection, or a discrete pulmonary nodule due to bronchogenic carcinoma. All were in the post-absorptive state without premedication.

Cardiac catheterization, with placement of the catheter tip in the main pulmonary artery, was done in the usual manner. The tracheo-bronchial double lumen tube, usually of the Carlens model, was then introduced under local anesthesia, immediately following the intravenous administration of atropine sulfate, gr. 1/150. The position of the tube was verified by fluoroscopy and the presence of leaks checked by attempting to force air from one spirometer into the other following inflation of the occluding bronchospirometric cuffs. The three unsuccessful attempts were due to improper placement of the bronchospirometry tubes, or to small leaks around the bronchial cuff, resulting in the incomplete separation of inspired and expired gas from the two lungs as demonstrated by analysis of expired gas. The indwelling needle was introduced into a brachial artery following the placement of both the cardiac catheter and the tracheobronchial double lumen tube.

Each inspired gas mixture was breathed by means of a separate open circuit. Each circuit consisted of a tank containing the specific mixture of oxygen in nitrogen, an anesthesia bag interposed between the reducing valve and a low-resistance, three-way valve connected with one of the two lumens of the tracheo-bronchial tube, and a collecting spirometer for measurement of minute ventilation ($\dot{V}_E$), and sampling of expired gas. During the control period, the normal lung breathed the hyperoxic mixture (F_{IO_2} = .25 or .33); the other lung breathed ambient air (F_{IO_2} = .21). After at least 10 minutes of equilibration, expired gas, arterial and mixed venous blood samples were simultaneously collected, over corresponding time periods, for determination of pulmonary blood flow. Following a second control period the low oxygen mixture (F_{IO_2} = .10 or .12) was substituted, without the patient's knowledge, for ambient air; the hyperoxic mixture to the "normal" lung was continued. After 15, and again, 20 to 25 minutes of re-equilibration, blood and expired gas samples were drawn for determination of pulmonary blood flows. In two patients, a final control study was repeated, using the initial gas mixtures, beginning approximately 10 to 20 minutes after cessation of unilateral hypoxia. Pulmonary arterial pressures were obtained using Statham strain gauge pressure transducers, a multi-channel apparatus with high sensitivity carrier amplifiers, and photographic recording of the cathode-ray images. Systolic and diastolic pressures were measured during two complete respiratory cycles and were averaged; mean pressures were obtained by planimetric integration of the corresponding areas.

Oxygen content and capacity of whole blood were determined by the Van Slyke-Neill apparatus; the fractions of oxygen and carbon dioxide in inspired and expired gas were determined by the 0.5 ml. micro-Scholander apparatus. All analyses were done in duplicate. From the analyses of inspired and expired gas, oxygen uptake, carbon dioxide output and respiratory exchange ratios were calculated for each lung and for both lungs.

TABLE II

Pulmonary blood flow and pulmonary arterial pressure during unilateral hypoxia

Subject	State	F_{IO_2} Lung H	F_{IO_2} Lung L	Pulmonary blood flow $\dot{Q}_H$ L./min.	$\dot{Q}_L$ L./min.	$\dot{Q}_T$ L./min.	$\frac{\dot{Q}_L}{\dot{Q}_T}\times 100$	Pulm. art. pressure s mm. Hg	d mm. Hg	m mm. Hg
H. J.	Control	.25	.21	3.38	2.79	6.17	45	20	10	15
	Hypoxic	.25	.12	3.47	3.25	6.72	48	19	8	14
D. C.	Control	.25	.21	4.18	3.78	7.96	47	27	14	20
	Hypoxic	.25	.10	4.27	4.32	8.59	50	26	14	20
G. H.	Control	.25	.21	3.87	2.98	6.85	44	24	9	15
	Hypoxic	.25	.10	3.07	3.03	6.10	50	25	9	16
P. T.	Control	.25	.21	4.17	5.79	9.96	58	18	8	14
	Hypoxic	.25	.10	3.94	5.67	9.61	59	19	7	15
J. S.	Control	.33	.21	4.73	3.10	7.83	40	16	8	13
	Hypoxic	.33	.10	5.43	2.91	8.34	35	19	9	14
R. R.	Control	.25	.21	3.63	3.70	7.33	50	21	9	15
	Hypoxic	.25	.10	3.69	3.85	7.54	51	19	8	14
Mean	Control			3.99	3.70	7.69	48	21	10	15
	Hypoxic			3.98	3.84	7.82	49	21	9	16

RESULTS

The data concerning the blood gas composition and the gas exchange in the lungs of the six subjects are presented in Table I. The figures listed for the control and unilateral hypoxia periods are the average of two determinations, except in subjects G. H. and J. S., where only one study was performed during unilateral hypoxia. The values for total pulmonary blood flow ($\dot{Q}_T$) and for the blood flow through each lung ($\dot{Q}_H$ and $\dot{Q}_L$) calculated from these data are presented in Table II, together with the corresponding pulmonary arterial pressures. Table III contains the time sequence of the individual studies, as well as the measurements of the total blood flow ($\dot{Q}_T$) and of blood flow through the hypoxic lung ($\dot{Q}_L$).

Control periods

During these periods, the mean arterial blood oxyhemoglobin saturation was 98 per cent, ranging from 97 to 99 per cent. Total minute ventilation ($\dot{V}_{E_T}$), as well as the ventilation of each lung ($\dot{V}_{E_H}$, $\dot{V}_{E_L}$) exceeded that observed in normal subjects under resting conditions.

TABLE III

Successive determinations of total blood flow, and blood flow through one lung prior to, and during unilateral hypoxia

Subject	State	Time of expos. *min.*	$\dot{Q}_T$ *L./min.*	$\dot{Q}_L$ *L./min.*	$\frac{\dot{Q}_L}{\dot{Q}_T} \times 100$
H. J.	Control	10	5.75	2.67	46
		15	6.58	2.91	44
	Hypoxic	15	6.56	3.05	46
		20	6.88	3.40	49
D. C.	Control	15	8.18	4.24	52
	Hypoxic	15	7.58	3.90	51
		25	9.60	4.74	49
	Control	15	7.76	3.33	43
G. H.	Control	10	6.78	2.49	37
		15	6.91	3.48	50
	Hypoxic	15	6.10	3.03	50
P. T.	Control	10	9.74	5.24	54
		15	10.18	6.34	62
	Hypoxic	15	9.88	5.72	58
		20	9.34	5.62	60
J. S.	Control	10	8.00	2.89	36
	Hypoxic	15	8.34	2.91	35
	Control	10	7.67	3.32	43
R. R.	Control	10	7.75	4.12	52
		15	6.90	3.27	48
	Hypoxic	15	7.35	3.55	48
		25	7.72	4.14	54

The total oxygen uptake ($\dot{V}_{O_{2T}}$) was elevated to a mean of 284 ml. per min., which corresponds to an average increase in metabolic rate of approximately 25 per cent over and above predicted basal values. The total carbon dioxide production ($\dot{V}_{CO_{2T}}$) was increased proportionally, and the average respiratory exchange ratio for both lungs (R_{E_T}) was 0.88. The lung receiving the hyperoxic mixture (lung H), usually the right lung, contributed 58 per cent of the total oxygen uptake and 57 per cent of the total carbon dioxide production.

The total cardiac output ($\dot{Q}_T$) was greater than normal, averaging 7.69 liters per minute, or 33 per cent above the predicted figure for normal resting individuals under standard metabolic conditions. The blood flow through the lung breathing ambient air ($\dot{Q}_L$) was 48 per cent of the total cardiac output. As seen in Table III, the differences between total blood flow during two control periods were small, regardless of whether the periods were in succession or separated by an intervening period of unilateral hypoxia. The largest difference between control periods was 14 per cent. The proportion of blood flowing through lung L $\left(\frac{\dot{Q}_L}{\dot{Q}_T} \times 100\right)$ was also constant, varying by less than 10 per cent in all but one patient (G. H.) where it reached 13 per cent.

Unilateral hypoxia

After exposure of lung L to the low oxygen mixture, the mean arterial oxyhemoglobin saturation decreased to 91 per cent. Neither the mean total minute ventilation ($\dot{V}_{E_H}$, $\dot{V}_{E_L}$), the total oxygen uptake ($\dot{V}_{O_{2T}}$), the total carbon dioxide production ($\dot{V}_{CO_{2T}}$), nor the production of carbon dioxide by each lung ($\dot{V}_{CO_{2H}}$), ($\dot{V}_{CO_{2L}}$) differed significantly from corresponding control values (Table I). However, the mean oxygen uptake of the low oxygen lung ($\dot{V}_{O_{2L}}$) decreased from 120 to 78 ml. per min., *i.e.*, from 42 to 27 per cent of $\dot{V}_{O_{2T}}$, and the oxygen uptake of the high oxygen lung ($\dot{V}_{O_{2H}}$) correspondingly increased from 164 to 218 ml. per min., or from 58 to 73 per cent of $\dot{V}_{O_{2T}}$. The constant carbon dioxide production in the face of diminished oxy-

gen uptake by the hypoxic lung (lung L) resulted in an augmented respiratory exchange ratio (R_{EL}) with a mean of 1.35; conversely, the hyperoxic lung (lung H) with a relative increase in oxygen uptake and constant carbon dioxide production, had a diminished mean respiratory exchange ratio (R_{EH}) of .66, as compared to the mean control R_{EH}, of .88.

In individual subjects, differences in total and unilateral blood flow between control and experimental periods were slight and inconsistent. Neither mean total blood flow ($\dot{Q}_T$) nor the partition of blood between the two lungs ($\dot{Q}_H$, $\dot{Q}_L$) altered during unilateral hypoxia (Table II). It is noteworthy also that in the four patients in whom two studies were made during unilateral hypoxia (Table III), the proportion of total blood flow through the hypoxic lung $\left(\frac{\dot{Q}_L}{\dot{Q}_T} \times 100\right)$, did not vary by more than 6 per cent for periods up to 25 minutes.

Pulmonary arterial pressures were recorded in all subjects. The tracings were characterized by marked respiratory swings. However, in sharp contrast with the results of studies in man, which demonstrate that the breathing of similar hypoxic mixtures by *both* lungs consistently cause pulmonary hypertension, pulmonary arterial systolic, diastolic and mean pressures remained at their control levels.

DISCUSSION

This investigation of the effects of unilateral pulmonary hypoxia upon ipsilateral pulmonary blood flow, has several aspects which merit further comment. They are discussed in the following order:

1) The validity of the use of the Fick principle for the measurement of the pulmonary blood flow under the particular experimental conditions of the study; 2) the validity of the assumption that pulmonary venous blood leaving the hyperoxic lung continues to be fully saturated with oxygen during the period of contralateral hypoxia; 3) the physiologic basis for (a) the reduction in oxygen uptake by the lung breathing the hypoxic gas mixture and (b) for the increase in oxygen uptake by the lung breathing the hyperoxic gas mixture, despite an unchanged distribution of pulmonary blood flow; 4) the site and mechanism of action of the hypoxic stimulus upon the pulmonary vascular bed; 5) a comparison of our results with those reported by others investigating the same problem in animals and man, and 6) the potentiality of this new method for the evaluation, in man, of the pharmacologic effects of various substances upon the blood flow through each lung.

1) *The validity of the use of the Fick principle in these experiments*

Potential limitations in the application of the Fick principle to the measurement of pulmonary blood flow during acute systemic hypoxia have recently been described (2, 3). These considerations, based primarily upon theoretical analyses of cyclic changes in respiration and circulation, have been put to experimental test (4, 5) and found to constitute only a slight source of potential error in a "steady state" of respiration and circulation. In particular, they have little applicability to the present study despite unilateral breathing of a mixture of 10 per cent oxygen in nitrogen, since (a) the degree of systemic arterial hypoxemia was slight, averaging 91 per cent oxyhemoglobin saturation during experimental periods, (b) the changes from control values, in mixed venous blood oxygen content and in total oxygen uptake, were small, and (c) variations from the control state of ventilatory, circulatory, and metabolic equilibrium, achieved during the stress of bronchospirometry, were at a minimum. The data show that even though the patients were not "basal" during the course of the consecutive control and hypoxic periods, the various cardio-pulmonary functions, including cardiac output, total minute ventilation, oxygen uptake, carbon dioxide output and respiratory exchange ratio for both lungs, remained stable, thus satisfying criteria previously suggested for the "steady state" (6). Account was also taken of the possibility that the injection of atropine sulfate, used in order to minimize bronchial secretions, could induce a changing state of the circulation, as the effect of the drug waxed and then waned during the course of successive measurements. However, tachycardia persisted unchanged during the entire procedure, and therefore a varying state of the circulation due to atropine sulfate, seems unlikely.

2) *Validity of the assumption that pulmonary venous blood leaving the hyperoxic lung is fully saturated with oxygen during the period of contralateral hypoxia*

A basic requirement of the measurement of blood flow through each lung is that pulmonary venous blood leaving the hyperoxic lung continues to be fully saturated with oxygen. Since, during the present experiments with unilateral hypoxia, the oxygen uptake by the hyperoxic lung increases whereas minute ventilation and carbon dioxide production remain essentially unchanged, the possibility exists that the oxygen content of pulmonary venous blood on the hyperoxic side might not reach 100 per cent of capacity, thereby obscuring a shift of blood from the hypoxic side. This possibility was tested by calculations based upon the experimental data; a sample calculation is presented for patient H. J.

a) In this patient, arterial blood P_{CO_2} = 40 mm. Hg during the control period, and remained unchanged during unilateral hypoxia.

b) Substituting in the alveolar gas equation the values obtained during the control periods, $R_{E_H} = R_{A_H} = .97$ (Table I), and $Pa_{CO_{2H}} = 40$ mm. Hg, it is found that $P_{A_{O_{2H}}} = 139$ mm. Hg.

c) Substituting then the values obtaining during unilateral hypoxia, $R_{E_H} = R_{A_H} = .75$ (Table I) and $Pa_{CO_2} = P_{A_{CO_{2H}}} = 40$ mm. Hg, it is found that $P_{A_{O_{2H}}} = 130$ mm. Hg. This tension is adequate for full saturation of pulmonary venous blood with oxygen.

d) However, the P_{CO_2} in the pulmonary venous blood leaving each lung may conceivably be different during the hypoxic period from the P_{CO_2} of the mixed arterial blood and thereby would alter the value for the $P_{A_{O_{2H}}}$ calculated above. For lung H, it is inconceivable that the $P_{PV_{CO_{2H}}}$, and therefore the $P_{A_{CO_{2H}}}$, should exceed the P_{CO_2} in mixed venous blood entering the lung, which in this case = 46 mm. Hg. Substituting, therefore, this latter value in the alveolar gas equation for lung H during contralateral hypoxia, it is found that $P_{A_{O_{2H}}} = 123$ mm. Hg instead of 130 mm. Hg.

These calculations indicate that the alveolar oxygen tension, applied to a particularly adverse experimental possibility, would nevertheless suffice to achieve full oxygen saturation of pulmonary venous blood leaving lung H during contralateral hypoxia.

Further calculations in the same patient show that a 25 per cent increase in blood flow through the hyperoxic lung during contralateral hypoxia, would result in an oxygen saturation in pulmonary venous blood from lung H of 95 per cent and a $P_{A_{CO_2}} = 76$ mm. Hg; the latter figure for alveolar P_{CO_2} is impossibly high since the P_{CO_2} of mixed venous blood = 46 mm. Hg.

In conclusion, there seems to be little doubt that under the conditions of these experiments, it is valid to assume that the oxygen saturation of pulmonary venous blood leaving the hyperoxic lung during contralateral hypoxia as well as during the control periods, is equal to the arterial oxygen capacity.

3) *The physiologic basis for diminished oxygen uptake and sustained carbon dioxide production by the hypoxic lung, and for the increased oxygen uptake and sustained carbon dioxide production by the hyperoxic lung*

The unchanged carbon dioxide production by each lung during unilateral hypoxia is not surprising, in view of the unchanged ventilation and pulmonary circulation. Of interest, however, is the mechanism responsible for the decrease in oxygen uptake by the hypoxic lung, and the augmented oxygen uptake by the hyperoxic lung, despite an unchanged distribution of pulmonary blood flow. Similar observations, during comparable degrees of hypoxia, have previously been made in man (1) and dog (7).

It is generally assumed that the rate of oxygen uptake at the mouth, in a steady state, measures the rate of diffusion of oxygen across the alveolar-capillary interface of lung, which is in turn equal to the product of the oxygen diffusing capacity of the lung ($D_{L_{O_2}}$) and the mean alveolar-pulmonary capillary oxygen pressure gradient (mean ΔP_{O_2}). During acute, but moderate hypoxia at rest, as long as cardiac output and ventilation do not change, the diffusing capacity of the lung is generally believed to remain constant (6, 8). The measured decrease in oxygen uptake by the hypoxic lung as well as the increase by the hyperoxic lung in the present experiments must then be due to corresponding change in the *mean* alveolar-pulmonary capillary oxygen pressure

gradient (mean ΔP_{O_2}). On the hypoxic side, the diminution in mean ΔP_{O_2} is largely due to the decreased $P_{A_{O_{2L}}}$, caused by the hypoxic inspired gas mixture. Conversely, the increased oxygen uptake on the hyperoxic side may in part be due to an increased initial alveolar-capillary P_{O_2} gradient caused by a decrease in $C\bar{v}_{O_2}$ during contralateral hypoxia; in accord with the discussion above, the degree of change in $P_{A_{O_{2R}}}$ is presumably slight but not available to direct sampling. The mechanism for widening the initial alveolar capillary P_{O_2} gradient is further indicated in Table I, which shows that the largest increase in oxygen uptake was associated with the greatest reduction in mixed venous blood oxygen content, and conversely, the increase in oxygen uptake was least in the patients with smallest decrease in mixed venous blood oxygen content.

The following calculations in patient H. J. illustrate and amplify the previous considerations:

For the application of the Bohr graphic integration method to the determination of mean ΔP_{O_2} and $D_{L_{O_2}}$ of the hypoxic lung, it is necessary to calculate a) the oxygen tension of blood leaving the pulmonary capillary bed; b) the mean alveolar oxygen tension; and c) the oxygen tension of mixed venous blood.

a) *The oxygen tension of blood leaving lung L* ($P_{PV_{O_{2L}}}$) *during hypoxia.* The data from which this value is derived are:

$\dot{Q}_L$ = 3.25 L. per min., $\dot{V}_{O_2}$ = 65 ml. per min., therefore,

$C_{PV_{O_{2L}}} - C\bar{v}_{O_2}$ (by Fick principle) = 2.0 ml. per 100 ml.

Since $C\bar{v}_{O_2}$ = 11.8 ml. per 100 ml., $C_{PV_{O_{2L}}}$ = 11.8 + 2.0 ml. = 13.8 ml. per 100 ml. and HB_{O_2} saturation of PV_L = 83 per cent. At pH = 7.40 using a standard oxyhemoglobin dissociation curve, $P_{PV_{O_{2L}}}$ = 49.0 mm. Hg.

b) *The mean alveolar oxygen tension* ($P_{A_{O_{2L}}}$). This value is calculated from the alveolar gas equation ignoring the Haldane effect and using $P_{a_{CO_2}}$ for $P_{A_{CO_2}}$ and R_E for R_A; (9) $P_{A_{O_{2L}}}$ = 51 mm. Hg.

c) *The mixed venous blood oxygen tension* ($P_{V_{O_{2L}}}$). The mixed venous blood HB_{O_2} = 72 per cent. At pH = 7.38, $P\bar{v}_{O_2}$ = 38.5 mm. Hg.

d) *The mean alveolar-pulmonary capillary oxygen tension gradient (mean* ΔP_{O_2}*) for lung L during hypoxia.* This value obtained by a modification of the Bohr graphic integration method (8) = 5.8 mm. Hg.

e) *The diffusing capacity* ($D_{L_{O_2}}$) *of lung L during hypoxia.* Since $\dot{V}_{O_2}$ = 60 ml. per min.,

$$D_{L_{O_2}} = \frac{60}{5.8} = 10 \text{ ml. per min. per mm. Hg.}$$

f) *The mean alveolar-pulmonary capillary oxygen tension gradient (mean* ΔP_{O_2}*) for lung L breathing ambient air.* Since neither blood flow nor minute ventilation changed during acute hypoxia, the $D_{L_{O_2}}$ is assumed to remain unchanged at the two levels of oxygenation. The $\dot{V}_{O_2}$ while breathing ambient air = 84 ml. per min.; the mean

$$\Delta P_{O_2} = \frac{84}{10} = 8.4 \text{ mm. Hg.}$$

g) *The mean alveolar-pulmonary capillary oxygen tension gradient (mean* ΔP_{O_2}*) for lung H.* Assuming the same $D_{L_{O_2}}$ for lung H and lung L, mean

$$\Delta P_{O_2} = \frac{137}{10} = 13.7 \text{ mm. Hg during control and } \frac{166}{10} = 16.6 \text{ mm. Hg during contralateral pulmonary hypoxia.}$$

The values for total $D_{L_{O_2}}$ = 20 ml. per min. per mm. Hg, and the A-a P_{O_2} gradient in lung L during hypoxia = 2 mm. Hg, are in accord with those previously described for normal subjects (9, 10).

4) *Site and mode of action of an hypoxic stimulus upon the pulmonary vascular bed*

In our experiments, the hypoxic stimulus, applied to one lung by airway, could have acted upon the conducting airway, alveoli, pulmonary capillaries and post-capillary vascular segments of the lung, to alter resistance to blood flow. Since there was no shift of blood flow from the hypoxic to the contralateral lung during unilateral hypoxia, there is no evidence to indicate an effect of a low oxygen partial pressure upon pulmonary vascular resistance to blood flow. Such an increase in vascular resistance has been postulated by Nisell (11, 12) who invoked: a *direct* effect upon the walls of the pulmonary

vessels mediated through the circulating hypoxic blood, and an *indirect* effect on these vessels due to the action of the hypoxic inspired gas mixture upon the intra-pulmonary elastic structures surrounding them. It is apparent that conclusions based on studies with the isolated, heart-lung preparations in dogs and cats are not readily applicable to intact man. Furthermore, in the present experiments, changes in mixed venous blood saturation failed to alter either total pulmonary blood flow or pulmonary arterial pressures, suggesting a negligible effect of acute hypoxia of moderate degree, mediated through the perfusing blood, upon the resistance to blood flow of the *precapillary* segments of the pulmonary circulation. This observation is further supported by other studies not yet published, but reported elsewhere in 1954 (13) which intend to compare the effect of exercise and of bilateral pulmonary hypoxia upon pulmonary blood flow and pulmonary arterial pressure. From these studies it appears that the degree of oxygen unsaturation in the mixed venous blood has no discernible effect upon the pulmonary vascular resistance in man.

Finally, these experiments do not contribute any information concerning the mechanism recently proposed by Aviado, Ling, Quimby, and Schmidt (14) on controlled perfusion experiments in animals, whereby the effects of acute hypoxia upon the pulmonary circulation were ascribed to stimulation of the carotid body by the hypoxic arterial blood, thereby eliciting vasoconstrictor impulses transmitted by the sympathetic nervous system to the pulmonary vessels. Since the degree of arterial hypoxemia in our studies on man was mild, and since the pulmonary arterial pressures remained unchanged, this hypothesis was not put to test. It is, however, pertinent that in the unpublished studies referred to above (13) surgical excision in man of a considerable portion of the sympathetic innervation of the lung, including both stellate ganglia, one mid-cervical ganglion, and the three upper thoracic sympathetic ganglia and anterior rami bilaterally, had no effect upon the degree of pulmonary arterial hypertension elicited by acute bilateral pulmonary hypoxia.

If systemic arterial hypoxemia does contribute to the elevation of pulmonary arterial pressures, mechanisms other than reflex vasoconstriction of the *pulmonary* vascular bed may be involved. For example, it is known that acute hypoxia induces vasoconstriction in the systemic circulation, excepting the cerebral and coronary segments. This systemic vasoconstriction suggests the possibility that increase in pulmonary artery pressure may be passive rather than active, involving (a) a shift of blood from the systemic to the pulmonary vessels, thereby causing an increased distension of the pulmonary vessels, and (b) an increased cardiac output (15) into the more distended pulmonary vascular bed. This possibility deserves further exploration.

5) *Comparison of present results with those of previous studies*

Our experiments have failed to demonstrate any significant variations in the minute blood flow through each lung during unilateral hypoxia. This is in accord with the conclusions of Jacobaeus and Bruce (1) who studied the problem in man with a method involving rebreathing in a bag, in order to equilibrate a gas mixture with the mixed venous blood, and a number of assumptions which they recognized as open to question. In animals, the results of investigations using rather indirect data have been inconsistent. Thus, in dogs, Moore and Cochran (16) could demonstrate no alteration in the per cent of the total cardiac output perfusing the hypoxic lung during unilateral hypoxia, whereas Rahn and Bahnson (7) calculated a prompt shift of blood flow from the hypoxic to the well-oxygenated lung. The latter studies were handicapped by lack of blood samples, and consequently required extrapolation from values for the respiratory gases in their gaseous phase to their corresponding concentrations in the blood phase. In rabbits, Dirken and Heemstra (17) on the basis of arterial blood oxygen saturation data, adduced evidence for a shift of blood flow from the hypoxic lung to the contralateral, well-oxygenated lung occurring only after 8 to 10 hours of continued unilateral hypoxia. It is, therefore, pertinent to emphasize, with respect to the possible influence of the duration of application to the hypoxic stimulus, that our studies during unilateral hypoxia did not last longer than 25 minutes.

In dogs, using more direct methods of measurement than in the animal experiments cited above, Atwell, Hickam, Pryor, and Page (18) observed a shift of blood flow away from the hypoxic lung in half of the animals within 20 minutes, whereas Peters and Roos (19) observed a shift at 25 minutes in 8 of 11 animals, with gradual return, in some instances, to the control, pre-hypoxic distribution of pulmonary blood flow as the experiments were prolonged. It is difficult to reconcile these conflicting results or to ascribe them to species differences, since there were great variations in (a) the experimental protocols; (b) the types and degrees of anesthesia; (c) the severity of systemic arterial oxyhemoglobin unsaturation, either present during the control periods, or developing during unilateral pulmonary hypoxia, and last, but not least; (d) the lack of attainment or maintenance of a steady state of circulation, respiration, and metabolism. The present experiments were designed to minimize the effect of these specific variables; they demonstrate that in man, during a stable state of respiration and circulation, in the absence of marked systemic arterial hypoxemia, a moderate degree of unilateral pulmonary hypoxia fails to alter pulmonary arterial pressures or flow. Experiments are in progress with the same method in order to test the effect of a greater degree of unilateral hypoxia, and *pari passu*, of a more marked reduction in systemic arterial oxyhemoglobin saturation.

6) *Applicability of the method to the study of other problems*

The method reported herein may be extended to problems other than that of the effects of unilateral pulmonary hypoxia. Among them one may mention 1) the influence of diseases of the lungs upon the partition of pulmonary blood flow and 2) the evaluation of the effects of pharmacodynamic agents, administered by air-way or blood stream upon the ipsilateral pulmonary vascular resistance. For such studies it would seem advantageous to administer to each lung an inspired gas mixture differing notably in oxygen concentration. This would tend to augment the differences between (a) the oxygen uptake of each lung, and (b) the oxygen content and the oxygen capacity of the arterial blood and therefore, minimize the analytic errors attending the application of the Fick principle. Furthermore, the detection of small leaks around the bronchospirometric cuffs, identified by analysis of expired gas and determination of respiratory exchange ratios would be greatly facilitated.

SUMMARY

1. The techniques of bronchospirometry, cardiac catheterization and cannulation of a peripheral artery have been combined for the application of the Fick principle to the determination of blood flow through each lung in man.

2. The essential features of the method are 1) the separate measurement, by collection of appropriate samples, of the oxygen uptake of each lung, and of the oxygen content of the mixed venous and of the arterial blood, and 2) the assumption that under the conditions of these experiments, the oxygen content of the pulmonary venous blood draining one lung is identical to the oxygen capacity of the arterial blood. This latter assumption was verified by (a) the demonstration of complete arterial blood oxygen saturation, when the subjects in these studies were breathing a mixture of 25 per cent or 33 per cent oxygen in nitrogen with both lungs, and (b) a theoretical analysis of experimental data.

3. During unilateral breathing of hypoxic gas mixtures capable of increasing pulmonary arterial pressure when applied to both lungs, no alteration in either total minute blood flow, or in the distribution of blood flow to each lung was observed. It is therefore concluded that the hypoxic stimulus does not act directly upon either the lung or the post-arteriolar segments of the pulmonary vascular tree to increase pulmonary vascular resistance to blood flow.

4. The physiological factors responsible for the differences in oxygen uptake by both lungs despite the maintenance of pulmonary blood flow during unilateral hypoxia, are discussed.

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STUDIES IN INTRAVASCULAR COAGULATION. III. THE PATHOGENESIS OF SERUM-INDUCED VENOUS THROMBOSIS [1, 2]

By STANFORD WESSLER with the technical assistance of KATHLEEN WARD and CAROL HO

(From the Medical Research Department of the Yamins Research Laboratory, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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The mechanisms involved in the initiation, propagation, and dissemination of intravascular clots remain obscure. The identification of each new clotting factor has raised the possibility that abnormalities in these factors might be found among patients with thromboembolism. Data have yet to be presented, however, demonstrating a causal relationship between intravascular thrombosis and an alteration in any of these clotting factors. Our efforts to uncover such a relationship proceeded from two observations. One was the impressive evidence that stasis plays an important role in the development of intravascular clotting. The other was the significant fact first established by Alexander, deVries, and Goldstein (1) and later confirmed by Owren (2) and Koller, Loeliger, and Duckert (3) that there is normally present in human serum a stable factor which accelerates prothrombin conversion. This factor has been variously called SPCA, Convertin® and factor VII.

It has been repeatedly stated that blood stagnating in a vein included between two ligatures will remain liquid for weeks (4, 5). We have found, however, that although clot formation was never observed in such vein segments of dogs within 20 minutes of their isolation, fibrin clots were always recognized in blood completely stagnant in a vein for 90 minutes (6). When first formed, all such clots were small, increased in size only slowly with time and were always incomplete for at least eight hours after isolation of the vein.

METHODS

To produce large clots rapidly, it was necessary to accelerate coagulation within these isolated vein segments. We selected serum to accomplish this because serum possessed clot promoting activity *in vitro*, was a natural product of the coagulation process itself, and had, like plasma, been successfully used as a blood substitute in man (7).

It soon became evident that a large thrombus could be regularly induced in the following manner. Dogs, 18 to 23 Kg. in weight, were anesthetized with Nembutal®. A segment of jugular vein three to six cm. in length was freed from its surrounding structures and its tributaries ligated. Canine serum from another animal was then infused into a distant antecubital vein. One minute later, after the infused serum had been dispersed throughout the circulation, the previously freed jugular segment was gently isolated with 27 mm. seraffine clamps.[3] A large red clot was routinely formed in the isolated vein within 60 seconds after clamping. To produce these clots, volumes of serum in excess of 90 cc. and isolation of the vein within two minutes after completion of the serum injection were required.

This technique demonstrated that in the dog the systemic infusion of heterologous canine serum induced a transient hypercoagulable state during which rapid massive thrombosis developed *in veins containing stagnant blood far removed from the site of injection.* Subsequent experiments have in part characterized the moiety in serum, the degree of stasis and the extent of endothelial damage required to precipitate such intravascular thrombi. In these experiments established methods were used to determine the overall one-stage plasma prothrombic activity (8), prothrombin (9), Ac-globulin (10), and SPCA (11) concentrations.

[1] This study was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-220 and by U. S. Public Health Grant No. H-1027. The work was done, in part, during the tenure of an Established Investigatorship of the American Heart Association.

[2] Presented in brief before (a) the Combined Meeting of the Panel on Blood Coagulation of the Subcommittee on Blood and Related Problems, National Research Council, Washington and the Panel on Coagulants and Anticoagulants, Defense Research Board, Ottawa, Canada on April 21, 1954 and (b) the Meeting of the American Society for Clinical Investigation, May 3, 1954.

[3] Manufactured by Arthur H. Thomas, Philadelphia, Pa.

RESULTS

The clot promoting activity of serum

Infusions of 100 cc. aliquots of physiologic saline solution, and of fresh plasma repeatedly failed to produce venous clots under the same conditions. These findings confirmed the specificity of serum for this reaction and indicated that the effect was not dependent upon a sudden increase in circulating blood volume or clotting factors present only in plasma.

Among the factors necessary for normal coagulation in man,[4] SPCA is found in appreciable amounts in serum stored for 24 hours at 4° C. Current knowledge does not permit definition of SPCA as one or more distinct substances. For the present, it can be described as an entity which —though distinct from Ac-globulin—is also essential for the physiologic conversion of prothrombin (12, 13). SPCA is present in plasma as a precursor called pro-SPCA (pro-convertin). During coagulation pro-SPCA is converted to SPCA (Convertin®). The *in vitro* tests used in this study, however, do not distinguish between the inactive and active forms but measure the total amount of SPCA complex present in a given sample (11). The failure of plasma to induce clotting in isolated venous segments suggests, however, that this phenomenon is not attributable to pro-SPCA but rather to its activated form, SPCA.

To determine the role of SPCA in serum-induced clots, SPCA-poor and SPCA-rich serum and serum fractions were tested. Dicumarol® is known to reduce the amount of circulating SPCA complex in addition to prothrombin (14). Serum was therefore obtained from dogs given Dicumarol® in which the SPCA content was less than one per cent of the control value. Infusions of 100 cc. of such sera repeatedly failed to induce intravascular clotting in stagnant blood. SPCA-poor and SPCA-rich serum fractions were obtained by the barium sulfate adsorption of normal canine serum.[5] Infusions of 100 cc. of the supernatant of the serum after barium sulfate adsorption, containing only trace amounts of SPCA, failed repeatedly to induce clotting in isolated vein segments. In striking contrast, the dialyzed eluate containing most of the SPCA activity of the parent serum, when infused in 25 cc. amounts into a distant antecubital vein, induced a large clot within the segment of jugular vein isolated within 30 seconds of the completion of the injection in 94 of 96 consecutive animals.

TABLE I

Canine SPCA values in serum and corresponding barium sulphate serum eluate (expressed in per cent of normal serum)

Serum	$BaSO_4$ eluate
60	162
94	198
98	198
123	312
124	430
78	198
100	280
48	205
114	234
98	186

Sera after barium sulfate adsorption were rich in SPCA (Convertin®) activity, having activities two to four times that of the parent serum (Table I). These eluates were free of demonstrable amounts of Ac-globulin and contained at most only trace quantities of thromboplastin, prothrombin, thrombin, fibrinogen and fibrin. Thus, when the eluate was substituted for dog brain thromboplastin in the one-stage prothrombin test, the plasma prothrombin time was 70 seconds instead of 20, a change representing a 1/500 dilution of thromboplastin. The prothrombin content (two-stage) of the preparation was less than one per cent of that in normal canine plasma. The thrombin content of the eluate was negligible: 0.1 cc.

[4] These include thromboplastin, antihemophilic globulin (AHG), plasma thromboplastin component (PTC), plasma thromboplastin antecedent (PTA), prothrombin, Ac-globulin, SPCA, thrombin, fibrinogen, and fibrin.

[5] Barium sulfate (Baker, C.P.) was added to serum previously stored for eight hours at 4° C. (100 mg. per cc. serum). The mixture was shaken well for five minutes, allowed to stand at 4° C. for eight hours, centrifuged at 2,000 rpm. for 20 minutes and the supernatant decanted. The barium sulfate was washed three times with 60 cc. 0.02 M acetate buffer (pH 4.98). To the washed barium sulfate was added a volume of 5 per cent sodium citrate equal to ⅛ of the original serum volume. This mixture was shaken well and stored for eight hours at 4° C. The eluate was recovered by centrifugation at 2,000 rpm. for 20 minutes and a second elution obtained with a second volume of citrate equal to the first. The combined eluates were dialyzed against running tap water for eight hours.

mixed with 0.1 cc. of either a standard fibrinogen solution or normal dog plasma did not induce a clot in tubes observed for 30 minutes (one unit of thrombin will clot a standard fibrinogen solution in 15 seconds).

Moreover, an infusion of 25 cc. of isotonic saline containing 300 to 600 units of bovine thrombin (Parke, Davis) or an infusion containing 25 cc. of a 1 to 10 saline dilution of dog brain thromboplastin[6] were required to induce clots in isolated segments similar to those initiated by 25 cc. of the serum after barium sulfate adsorption. Slightly larger amounts of thrombin and thromboplastin produced rapid and extensive intravascular thrombosis frequently killing the animal.

In brief, these observations suggest that the effectiveness of serum in producing intravascular thrombosis may be intimately related to the SPCA activity of the preparation. New factors essential to normal coagulation are, however, still being discovered. Some evidence has been uncovered in our own laboratory indicating that SPCA is not the sole factor responsible for this phenomenon (15).

The role of stasis

The degree of stasis required to induce a clot was investigated in the following manner. In 14 dogs single silk ties were passed under the right jugular and femoral veins. To reduce trauma, the vessels were not freed from their surrounding structures. The barium sulfate eluate of serum was then infused through an antecubital vein. Within 30 seconds after completion of the infusion the right jugular vein was narrowed[7] and the right femoral vein occluded by the silk ties. Fifteen minutes later, after the clot-promoting effect of the eluate had been entirely dissipated (15) the left jugular and femoral veins were similarly treated. Examination one hour later revealed that half of the jugular veins which were narrowed and all femoral veins which were occluded within 30 seconds after the injection contained large nonadherent thrombi distal to the sites of obstruction. In none of the veins narrowed or occluded 15 minutes *after* the injection was a single clot found. The evidence clearly indicates that whereas local stasis alone was uniformly inadequate to induce thrombosis, the combination of local stasis and systemically infused SPCA-rich serum fractions promptly triggered clot formation at these sites of local obstruction. Moreover, stasis did not have to be complete, although complete obstruction increased the incidence and size of the clot.

Role of endothelial damage

The extent to which endothelial injury may contribute to this experimental induction of intravascular thrombosis is difficult to evaluate. From the above and from earlier observations (16) it is clear that narrowing *per se* does not produce enough endothelial damage to induce thrombosis by the method used. Moreover, histologic study of vein segments examined one to 43 days after venous narrowing showed no evidence of intimal damage or superimposed thrombosis. Such histologic observations, of course, do not exclude morphologic injury (17). The absence of significant endothelial injury was further suggested, however, by the repeated observation that clots arrested at such narrowed areas did not become adherent to the underlying vein wall until after the fourth day (16). Finally, in a series of 16 dogs the jugular veins were obstructed by gentle manual pressure or by tourniquets inflated to a pressure of 40 mm. of mercury immediately after the infusion of the serum eluate. Although in none of these animals were the veins isolated or the skin incised until after the effect of the infusion had been dissipated, grossly visible thrombi were demonstrated distal to the obstructed zone in half of the animals.

DISCUSSION

In man it has been suggested that SPCA may play a role in thromboembolism (14, 18). Several investigators have, in fact, demonstrated a temporary rise in the SPCA complex of human pregnant and post-partum plasma (19–22). No correlation has yet been made, however, between

[6] Thromboplastin extract of dog brain as prepared in the usual manner for one-stage prothrombin determinations.

[7] Approximately 75 per cent narrowing of the lumen was accomplished by placing a metal probe (0.6 by 2.5 mm.) alongside the vein and tying the ligature snugly around both the probe and vein: the probe was then immediately withdrawn.

these increased levels of circulating SPCA complex and the occurrence of thromboembolism.

The studies herein described provide the first direct experimental evidence that, in the dog, the infusion of canine serum or serum fractions rich in SPCA may initiate venous thrombosis at sites of partial or complete stasis. Significant endothelial reaction has not been implicated as a factor in this reaction, and in any event is similar in all control situations where thrombi do not occur. It is well known that retarded local venous flow and the production of serum are common to the postoperative and post-partum states. It is therefore proposed as a working hypothesis that the pathogenesis of venous thrombosis in these clinical conditions may be referable to local venous stasis combined with a transient increase in SPCA activity or in some other entity produced during coagulation, present in serum and exhibiting the characteristics already described. Further purification of pro-SPCA and SPCA and improved methods for their specific measurement will aid in determining the validity of the proposition.

In addition to affording some insight into a mechanism whereby intravascular thrombosis may actually be induced in man, the technique herein described provides a new experimental approach to the multifaceted problems of thromboembolism. In the laboratory it has already afforded a unique method for comparing the efficacy of various anticoagulant and lytic agents recommended in the treatment of thromboembolism (23, 24). The method has also provided an additional tool for the *in vivo* investigation of venous thrombosis and pulmonary embolism.

SUMMARY

The technique herein described provides a new experimental tool for the investigation of many problems in the field of thromboembolism. The systemic infusion of canine serum or its $BaSO_4$ eluate produces, in the dog, massive intravascular thrombosis at localized sites of retarded or obstructed venous flow. Significant endothelial injury need not be involved in this reaction. It is proposed that the pathogenesis of venous thrombosis in the postoperative and post-partum states may be referable to a transient increase in SPCA activity or some other as yet unidentified entity in serum together with local venous stasis.

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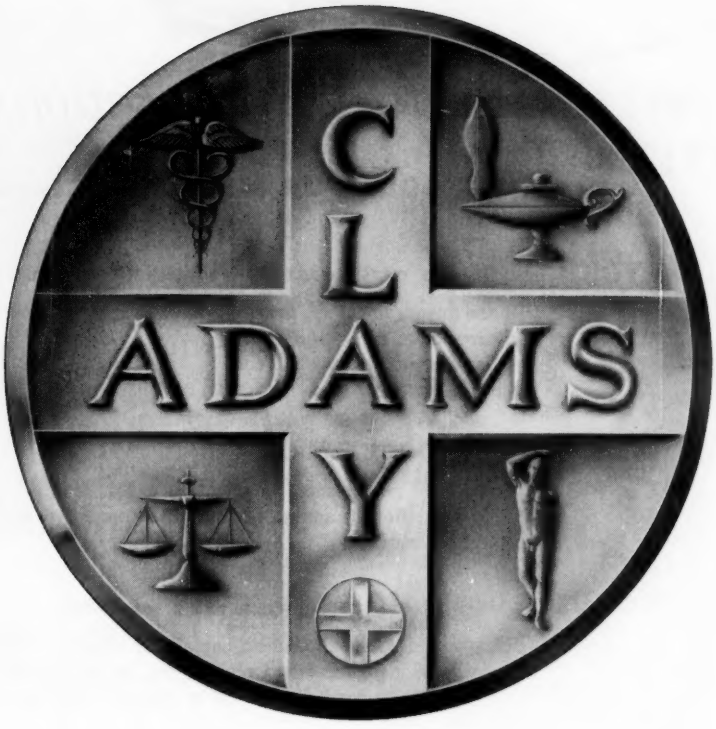

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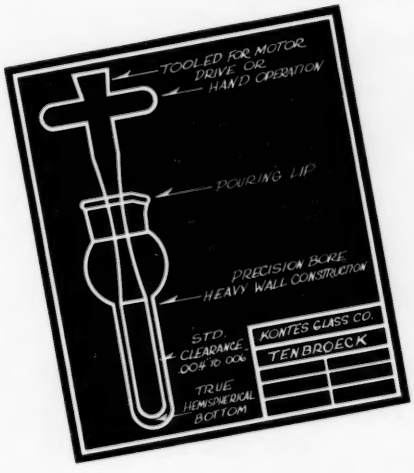

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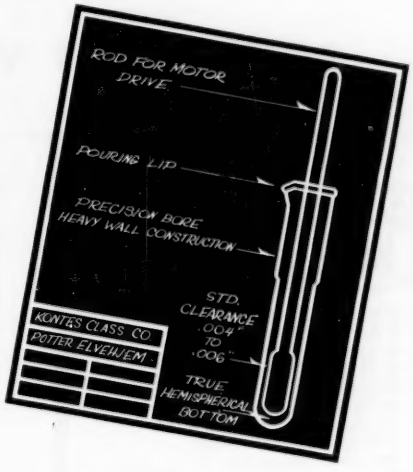

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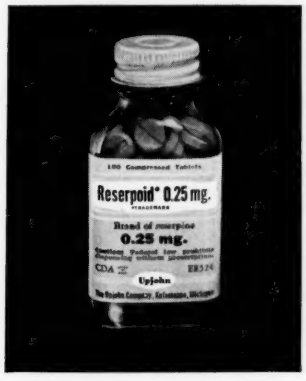

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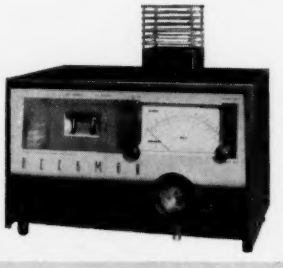

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